Identification of Genes in the Dorsal Raphe Nucleus Regulated by Chronic Stress and Citalopram

Ph.D. Thesis

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Submitted by

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Declaration:

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no materials previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university or other institute of higher education, except where due acknowledgment has been made in the text.

Signature

Name

Date and place

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I have to say 'thank-you' to my family and all my friends, wherever they are. Much of appreciation goes to Mom and Dad, and then the rest of my family; their support and belief in me have always inspired me in my endeavours. Last, but certainly not least, I thank my wife, my first and only love Xiao Yang, whose love gave me the strength and encouragement to follow the path that I felt was right, and whose patience and unselfishness allowed me the freedom to complete the task. This dissertation is lovingly dedicated to all those people.

Harry Harlow's (1905-1981) opinion regarding the use of animal models in the study of psychiatric disorders: *"You have to be crazy to use animal models for the study of human psychopathology", and then he added: "you have to be crazy if you do not because of all the knowledge that is gained from work with animals".*

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Abbreviations

CBP:	CREB-binding protein
CIT:	Citalopram
DCIT:	Demethylcitalopram
DRN:	Dorsal raphe nucleus
EST:	Expressed sequence tag
FST:	Forced-swimming test
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HAT:	Histone acetyltransferase
HPA axis:	Hypothalamus-pituitary adrenal axis
5-HT _{1A} receptor:	5-Hydroxytryptamine 1A receptor
5-HT:	5-Hydroxytryptamine (serotonin)
NDRG2:	N-myc downstream-regulated gene 2
NSE:	Neuron-specific enolase
PAGE:	Polyacrylamide gel electrophoresis
PCR:	Polymerase chain reaction
SDS:	Sodium dodecyl sulfate
SERT:	Serotonin transporter
SH:	Subtractive cDNA hybridization
SNAP-25:	Synaptosomal associated protein-25 kD
SSRI:	Selective serotonin re-uptake inhibitor
SV2b:	Synaptic vesicle glycoprotein 2b
TCA:	Tricyclic antidepressant
TPH:	Tryptophan hydroxylase

Summary

It is known that stress, especially when it is chronic, can lead to mood disorders and changes in the serotonergic (5-HT) system, which most probably play a role in stress-induced neuropathologies. Neurochemical measures indicated that serotonergic neurons in the dorsal raphe nucleus (DRN) are activated by stress. Clinical studies have shown that the therapeutic effect of antidepressants, including specific serotonin re-uptake inhibitors (SSRIs), occurs only after 2-3 weeks of chronic treatment. This lag phase may indicate that antidepressants change central nervous gene expression in a time dependent manner, however, little is known about such processes in stressed subjects. The aim of the present thesis was to identify genes in the rat DRN that are regulated by chronic social stress and to investigate whether these genes are regulated by the selective SSRI citalopram.

The first part of the thesis describes the identification of genes that are differentially expressed by chronic social stress in the DRN. Using a resident intruder paradigm, male Wistar rats were chronically stressed by daily social defeat during 5 weeks. RNA was isolated from their DRN, cDNA was generated, and subtractive cDNA hybridization was performed to clone sequences that are differentially expressed in the stressed animals. From the cDNA libraries that were obtained, the following genes were selected to quantify mRNA expression using quantitative real-time PCR: Two genes related to neurotransmission, synaptosomal associated protein-25 (SNAP-25) and synaptic vesicle glycoprotein 2b (SV2b); a glial gene presumptively supporting neuroplasticity (N-myc downstream-regulated gene 2, NDRG2); neuron specific enolase (NSE) that is known to promote neuronal growth and survival; and a gene possibly related to stress-induced regulation of transcription, CREB binding protein (CBP). Genes directly related to 5-HT neurotransmission could not be identified by subtractive cDNA hybridization which may suggest that their expression is not regulated by stress. However, quantitative real-time PCR was nevertheless performed for the serotonin transporter (SERT), the 5-HT_{1A} autoreceptor and the tryptophan hydroxylase (TPH) genes 1 and 2. It was found that only TPH1 mRNA was upregulated by chronic stress. These data reveal that 5 weeks of daily social defeat leads to significant changes in the expression of genes related to neurotransmission, neuroplasticity and 5-HT synthesis in the DRN,

whereas expression of genes directly related to 5-HT release is apparently normal after this period of chronic stress.

The second part of the thesis was designed to investigate the impact of a chronic citalopram treatment (30mg/kg/day, for 4 weeks administered via drinking water) on the mRNA expression of the above described genes in the DRN. The appropriate dose of citalopram was determined in a pilot study. Real-time PCR showed that citalopram normalized the stress-induced upregulation of mRNA for three genes: SV2b, CBP and NDRG2. The SSRI had no significant effect on SNAP-25 mRNA, but upregulated the expression of NSE mRNA in both stressed and unstressed animals. The potential impact of citalopram on the genes directly related to serotonin transmission was also investigated. It was found that citalopram reduced 5-HT_{1A} autoreceptor mRNA expression only in stressed animals. TPH 1 and 2 genes respond differentially to citalopram. The expression of TPH1 mRNA was normalized only in the stressed animals, whereas TPH2 mRNA was reduced in all treated subjects. These findings demonstrate that in the DRN of chronically stressed rats, citalopram restores mRNA expression of distinct genes involved in neurotransmitter release/ neuroplasticity and 5-HT biosynthesis.

To analyze whether stress and citalopram also change protein expression in the DRN, Western blot experiments were performed. These experiments revealed that chronic stress increased the expression of SV2b, SNAP-25, NSE, and TPH protein. Citalopram reversed the stress-induced upregulation of SV2b but had no significant effect on the amount of SNAP-25 protein. Furthermore, expression of syntaxin 1A and synaptophysin protein was not affected by either stress or citalopram. This indicates that stress and citalopram have no global effect on all synaptic/synaptic vesicle proteins in the DRN but that only distinct genes are affected. Stress-induced upregulation of NSE protein was not reversed by the SSRI but instead, citalopram enhanced NSE expression in control animals. The stress-induced upregulation of TPH protein correlated with enhanced expression of TPH1 mRNA, whereas antidepressant-induced normalization of TPH protein expression appears to be due to the reduction in TPH 1 and 2 mRNA.

To investigate whether the effects of stress and citalopram on gene expression are confined to the DRN, protein expression was also determined in the hippocampal formation. Data indicated that also in this brain region NSE expression is involved in stress-induced processes, however, citalopram had no effect on hippocampal NSE expression. In contrast to the DRN, hippocampal syntaxin 1A protein was upregulated by stress and normalized by citalopram.

In conclusion, the present data demonstrate that chronic stress upregulates the expression of distinct genes involved in neurotransmitter release/neuroplasticity which possibly reflect enhanced neuronal activity. The SSRI citalopram normalizes expression of some of these genes. Furthermore, the changes in gene expression are specific for the DRN indicating a regional effect of stress and citalopram, respectively. The stress-induced upregulation of TPH may reflect enhanced activity of 5-HT neurons, while the SSRI normalized TPH expression. One may speculate that similar mechanisms may contribute to the therapeutic actions of citalopram in patients.

1 Introduction

1.1 *Stress and mood disorders*

Stress was originally thought to have a merely endocrine character, and noxious stimuli of physical or chemical nature were primarily discussed in early days as stress factors. Subsequent studies demonstrated that the most potent stimuli for the hypothalamus-pituitary-adrenal (HPA) system are psychological stressful events (Mason, 1968). Since then, great progress has been made to understand the biology of stress reactions along with the behavioural and physiological alterations. In this body of research it was concluded that stress is not necessarily a harmful or a pathological factor to be avoided. Only when chronic and when the adaptive mechanisms can not be recruited, stress will result in a number of deleterious effects and health might be endangered. One potential consequence of uncontrollable, chronic stress is the dysregulation of activity of those central neurotransmitter systems implicated in anxiety and depression such as serotonin (5-HT) and noradrenaline (Anisman and Zacharko, 1992; Flügge, 2000). Chronic stress has been shown to activate the 5-HT neurons in the dorsal raphe nucleus (DRN) and to increase 5-HT levels in DRN target areas and within the nucleus itself (Maswood et al., 1998; Amat et al., 2005) which most likely impairs information processing in its diverse terminal regions, possibly leading to depression and other mood disorders. Stress also activates the HPA axis, meaning that release of glucocorticoid hormones from the adrenal cortex is increased. These corticosteroids are potent modulators of cell physiology and behaviour and the HPA axis serves as an alert system for the organism to react to environmental or physiological challenges (de Kloet et al., 1998). As demonstrated in animal studies, chronic stress leads to impaired feedback inhibition of glucocorticoids release that is in part due to decreased expression of hippocampal corticosteroid receptors. Hyperactivity of the HPA axis is also characteristic for depressive symptomatology in humans (Holsboer et al., 1984). This impairment of the HPA axis may have negative effects on brain regions involved in emotional processes, such as the prefrontal cortex, which modulates the activity of 5-HT

neurons in the DRN (Celada et al., 2001), which is likely to impair control belonging to the cognitive systems on 5-HT neurons, leading to increased risk for developing mood disorders.

Mood disorders, including major depression, represent a collection of psychological, behavioural and physiological symptoms whose frequency and chronicity constitute a recognizable clinical condition (DSM-IV, 1994). Despite intensive investigations, the exact neuropathological processes that lead to depressive diseases remain unknown. Stress appears to increase the risk of depression especially in the presence of a genetic predisposing factor (Kendler et al., 1999; Fava and Kendler, 2000; Caspi et al., 2003). It has been suggested that stress induces adaptive changes that enable the system to respond effectively to a potential life or social status threat. However, repeated uncontrollable stress may result in severe maladaptations and may lead to depressive disorders (McEwen, 2002; Fuchs et al., 2004a). Therefore, many experimental stress paradigms have been developed, which use chronic stress to induce depressive-like symptoms in animals (Dews, 1958; Willner et al., 1995; Willner, 1997; Fuchs and Flügge, 2002; Rygula et al., 2005), although one should bear in mind that psychiatric disorders are very complex and that not all aspects of these diseases can be modelled in experimental animals. However, the use of animal models to understand the neurobiology of those disorders is still a valuable tool.

Indeed some of those animal models provided evidence for their success. For instance the psychosocial stress model of the non-rodent species tree shrew, which is regarded as a valid model for depression, is a powerful tool to study the behavioural, endocrine and neurobiological changes that may underlie stress-related disorders such as major depression (Fuchs and Flügge, 2002; van Kampen et al., 2002). There is increasing evidence that the neurochemical aspects of psychiatric disorders are accompanied by neurobiological alterations in neurons and/or glial cells. Hence, morphological changes across various limbic and non-limbic circuits in the brain of depressed patients have been demonstrated (Manji et al., 2001; Manji and Duman, 2001). Moreover, post-mortem studies revealed a decrease in glial density in cortical and limbic areas (Rajkowska, 2000). Chronic psychosocial stress in tree shrews induced structural plasticity in the hippocampus, hippocampal volume loss and changed glial structures

(Czeh et al., 2001; Czeh et al., 2005; see Fuchs et al., 2004a, b). Besides the enrichment of our knowledge about behavioural, physiological and brain structural changes induced by stress, animal models have provided evidence for molecular modulatory changes, represented by changes in gene expression that may underlie stress-induced psychopathologies. Moreover, a remarkable progress towards understanding the molecular mechanisms behind antidepressant action has been achieved using animal models. For instance, among different parts of the brain known to be involved in the stress response, the hippocampal formation is the most extensively studied in terms of both chronic stress and antidepressant treatments (Alfonso et al., 2005). Based on several studies from the hippocampus it is well established that stress/antidepressant treatment modulate the expression of numerous genes related to distinct functions: neuronal growth/differentiation and spine related genes, transcription factors and their co-activators, signal transduction pathways, stress hormones, neurotransmitter systems, synapses components, cytokines and glial genes (Alfonso et al., 2005). Surprisingly, although the DRN has long been implicated in affective disorders (Gold et al., 1988; Meltzer, 1989), is known to be activated by stress (Chaouloff, 2000) and is the primary target of the most effective antidepressants (e.g. selective serotonin re-uptake inhibitors, SSRIs, see 1.2.2), little attention has been paid to the molecular and neuroplasticity mechanisms that are presumptively induced by stress and/or antidepressants in this nucleus.

1.2 *Dorsal raphe nucleus*

Disturbances in the 5-HT system are associated with affective disorders (Gold et al., 1988; Meltzer, 1989; Nemeroff, 1998). The 5-HT system originates in the raphe nuclei, an area that can be divided into two major parts: the cranial and the caudal divisions. The cranial nuclei contain the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN), and both project mainly to forebrain regions (Pineyro and Blier, 1999). The caudal nuclei include the raphe pallidus, magnus and obscurus that project to the brainstem and spinal cord (Deutch and Roth, 1999).

The DRN is the largest nucleus of the brainstem 5-HT nuclei containing about 50% of the serotonergic neurons in the rat CNS (Wiklund and Bjorklund, 1980). Approximately one third of the neurons in the DRN are serotonergic (Descarries et al., 1982). In the DRN, 5-HT release from the 5-HTergic neurons occurs at neuronal synaptic terminals and extrasynaptically within the DRN. Vesicle-containing dendrites (Descarries et al., 1982), dendro-dendritic synapses (Chazal and Ralston, 1987) and collaterals of serotonergic axons have been observed in the DRN (Beaudet and Descarries, 1981). It has been shown that upon neuronal stimulation the 5-HT level in the DRN is double as high as the level in the target areas that contain the serotonergic nerve terminals (see Pineyro and Blier, 1999). It is known that 5-HT neurons in the midbrain exhibit a spontaneous slow (1-5 spikes/s) and regular discharge. However, this firing rate is not constant, it varies from maximum firing ability during aroused state to become almost silent during rapid eye movement (REM). This firing rate was shown to be regulated by 5-HT receptors such as the 5-HT_{1A} (somatodendritic autoreceptor, which is regarded as a target for many drugs and linked to stress-related disorders; Chaouloff, 2000; Pineyro and Blier, 1999) and the 5-HT_{1B} receptor (more concentrated on the terminals; Pineyro and Blier, 1999). Following its release, 5-HT is actively taken up from the synaptic cleft by a high-affinity transporter located presynaptically (also located on cell bodies), the serotonin transporter (SERT). This molecule is of particular importance since it is the target for many therapeutically effective antidepressants, in particular the selective serotonin re-uptake inhibitors (SSRIs), and the less specific tricyclic antidepressants (TCAs, Pineyro and Blier, 1999).

1.2.1 Effects of stress on the dorsal raphe nucleus

Several data indicate that stress activates serotonergic (5-HT) neurons in the DRN. This activation increases 5-HT release in the DRN and in target areas (Palkovits et al., 1976; Kirby et al., 1997; Maswood et al., 1998; see Chaouloff, 2000). The molecular changes that may occur at the gene expression level and that may underlie this stress activation of 5-HT neurons are still unknown. Electrophysiological, molecular and biochemical studies trying to explain this activation mainly focused

on genes related 5-HT release. For instance, using electrophysiological approaches, restraint stress followed by social isolation as well as eight weeks of ultra-mild stress lead to desensitization of 5-HT_{1A} autoreceptors (Laaris et al., 1999; Lanfumey et al., 1999). A molecular study in another species, the male tree shrew, revealed no effect of chronic social stress on 5-HT_{1A} autoreceptor number in the DRN (Flügge, 1995). On the other hand, acute immobilization stress did not affect serotonin transporter (SERT) mRNA in the DRN (Vollmayr et al., 2000). Repeated immobilization stress upregulates expression of tryptophan hydroxylase 1 (TPH1) mRNA and protein in the rat DRN (Chamas et al., 1999; 2004). Tryptophan hydroxylase is the rate limiting enzyme required for 5-HT synthesis from the amino acid tryptophan. These data are supported by previous findings showing that chronic stress increases serotonin synthesis and turnover (see Flügge, 2000). Stress-induced activation of TPH activity was also addressed earlier (Azmitia and McEwen, 1974). However, recently, mice genetically deficient for TPH1 (tph -/-) had normal 5-HT levels in the brain stem and exhibited no significant behavioural deficits, and it has been shown that in the midbrain 5-HT system another gene called TPH2 plays a major role in 5-HT synthesis beside TPH1 gene (Walther et al., 2003; Zhang et al., 2004). These data may question previous results regarding the role of TPH1 gene in the increase in TPH protein expression in response to stress. The role of TPH2 gene in stress responses is still unknown.

Based on these data, the present thesis addresses two questions: (1) Is the expression of genes involved in synaptic neurotransmission modulated by stress? (2) In the light of the recent data on TPH genes 1 and 2, does stress change the expression of TPH 1 or 2 mRNA and protein? (see also 1.5).

1.2.2 Effects of antidepressants on the dorsal raphe nucleus

Since the monoamine hypothesis of depression was proposed about 40 years ago (Schildkraut, 1965), this hypothesis was supposed to explain the biological basis of affective disorders assuming that deficiencies or imbalances in the serotonin, noradrenaline and presumably dopamine systems underlie depressive-like neuropathologies. This hypothesis was supported by the efficacy of many drugs that elevate extracellular monoamine levels such as TCAs, or drugs that inhibit monoamine turnover,

monoamine oxidase inhibitors (MAOI). In spite of their therapeutic effects, those drugs were associated with many side effects due to their non-specific actions on monoaminergic systems. More selective drugs have been developed later showing more efficient therapeutic effects and less side effects such as the selective serotonin re-uptake inhibitors (SSRIs, e.g. citalopram, CIT) and noradrenaline re-uptake inhibitors (NRIs). However, recently some atypical antidepressant drugs (e.g. tianeptine) have been shown to bear antidepressive effects without acting in the classical way that fits into the monoaminergic hypothesis (McEwen et al., 2002; McEwen and Chattarji, 2004). Moreover, the monoamine hypothesis does not solve the puzzle of the lag phase (2-4 weeks) in the therapeutic action of SSRIs and other antidepressants. This supports an emerging hypothesis suggesting that the treatment of depression is likely to involve plasticity of neuronal pathways by acting on gene transcription (Duman et al., 1997; Nestler, 1998; Duman et al., 1999). Recent theories suggest that in general antidepressants, after chronic administration, may exert their therapeutic effects at the molecular level by reversing system dysfunctions, enhancing adaptive responses or by independently stimulating adaptive neuronal plasticity (Duman et al., 1997; Duman et al., 1999). Therefore, many studies were performed to explore potential effects of chronic antidepressant treatments on gene expression (Landgrebe et al., 2002; Newton et al., 2003; Wong et al., 2004). However, those studies did not focus on the DRN as an important regional target of antidepressants.

Investigations of chronic antidepressant treatments on gene expression in the DRN were performed addressing expression of 5-HT related genes. No effects of different antidepressants including CIT on mRNA expression for SERT, different 5-HT receptors including subtype 1A, and TPH in brains of rats treated chronically for up to 32 days were observed (Spurlock et al., 1994). However, 5-HT_{1A} mRNA was significantly decreased after 2-3 weeks of fluoxetine treatment in the anterior raphe of rats (Le Poul et al., 2000) and SERT mRNA was transiently decreased in the DRN of rats treated with fluoxetine after 7 days of treatment, but this effect disappeared after 21 days (Neumaier et al., 1996). Other studies showed that there are adaptive changes in 5-HT_{1A} autoreceptors (Kreiss and Lucki, 1995; Le Poul et al., 1995; El Mansari et al., 2005) and SERT (Lesch and Beckmann, 1993) induced by antidepressant

treatments, but these alterations did not occur at the mRNA level. Furthermore, upregulation of TPH1 gene and TPH protein expression and serotonin synthesis after two weeks of sertraline treatment has been reported (Kim et al., 2002). However, an effect of antidepressants on TPH2 gene has not yet been investigated.

It is surprising that our knowledge about the action of antidepressants on gene expression in the DRN is still poor, particularly in comparison to the knowledge gained from other regions such as the hippocampal formation. Furthermore, a confounding factor of all of those studies on the cellular and molecular effects of antidepressants, investigating the DRN and other brain regions, was that the experiments were performed on normal “non-stressed” animals. From clinical studies, it is known that antidepressants have not much effect in healthy subjects, but do work effectively in depressed patients. One has to assume that in healthy experimental animals, a potential antidepressant-induced reversal of presumed disease-related changes in gene expression towards normal cannot be observed. This idea is supported by the fact that in previous studies from our group (see e.g. Czeh et al., 2001; van der Hart et al., 2002) several neurostructural changes observed in stressed animals treated with antidepressants were not seen in the non-stressed animals representing the control group. Therefore, the experiments in the present thesis were designed to investigate and compare the impact of the SSRI CIT on selected stress-upregulated genes and serotonin related genes in “stressed” and “non-stressed” animals.

1.3 *The resident-intruder stress paradigm*

Since the concept of the 'animal model' was introduced into preclinical research, several experimental models have been used for the investigation of neurobiological mechanisms of psychopathologies and for the screening of new antidepressant drugs (Dews, 1958; Sarter and Bruno, 2002; Willner and Mitchell, 2002). However, little attention has been paid to social factors that are presumed to play an important role in many cases of human depressive disorders (Kessler et al., 1985; Kessler, 1997). It was suggested that in studies aimed at the analysis of stress-related psychopathologies, the use of naturalistic psychosocial stressors may represent an advantageous research strategy (Kessler et al., 1985; Gilbert and Allan, 1998; Gilbert et al., 2002). Social stressors are more likely to elicit stress response patterns similar to those observed in humans following stressful life events (Bjorkqvist, 2001). A naturalistic stressor, prevalent in real-life situations, is social defeat by an aggressive individual leading to subordination stress (Bjorkqvist, 2001). Social stress models are advantageous over other animal models that require aversive physical stimuli such as electric foot shock, restraint, water or food deprivation, or cold exposure.

In humans, loss of rank and/or social status is an example of loss experience, which is recognized as a specific type of life-events associated with a greater risk of depression (Brown, 1993). In the resident-intruder paradigm, under experimental conditions, social defeat can be obtained (Koolhaas et al., 1997; Miczek et al., 2004). In this paradigm, an adult male (the intruder) is introduced into the home cage of an unfamiliar, aggressive conspecific male (the resident). Animals interact rapidly, fight, and the intruder usually loses the encounter. Chronic social defeat in a non-rodent species, the tree shrew, has been shown to elicit a broad spectrum of behavioural and physiological correlates of human depression (Fuchs and Flügge, 2002). Also in rodents, a single social defeat by an aggressive male produces a variety of behavioural and physiological alterations (Koolhaas et al., 1990; Meerlo et al., 1996; Buwalda et al., 1999). Other studies analysing the effects of chronic social stress focused mainly on mice (Keeney and Hogg, 1999; Bartolomucci et al., 2004).

We have recently described a new rat model of chronic social stress (Rygula et al., 2005). In this model, male rats subjected to daily social defeat for a period of 5 weeks showed behavioural and physiological alteration such as reduced sucrose preference, increased immobility time in the forced-swimming test (FST), decreased locomotor and exploratory behaviour, increase in the adrenals weight and reduction in body weight gain (Rygula et al., 2005). Chronic CIT treatment ameliorated the stress-induced behavioural deficits (Rygula et al., 2006). In addition, chronic fluoxetine had the same effect, whereas treatment with the anxiolytic diazepam and the antipsychotic haloperidol did not reverse the stress-induced effects (Rygula et al., unpublished data). To assess the validity of an animal model, it has to fulfil at least two of three criteria: Face validity, predictive validity and construct validity (Willner and Mitchell, 2002). The fact that chronic social stress triggers symptoms resembling depressive symptoms in humans is regarded as face validity. On the other hand, the effectiveness of CIT and fluoxetine treatment in reversing the stress-induced behavioural disturbances can be taken as an argument for the predictive validity of the present paradigm as a model of depression. Finally, the model may have construct validity based on the fact that social and psychological factors play an important role in many cases of human depressive disorders. Therefore, chronic social stress in rats can be regarded as a valuable tool for the present thesis, to investigate the stress-induced effects on gene expression in the brain, with the aim to get an insight into molecular mechanisms that may underlie stress-related psychopathologies.

1.4 Analysis of gene expression

Different approaches can be employed to identify and investigate genes that are regulated by certain experimental condition such as stress or drug treatment: mRNA measurements of specific genes selected according to their biological functions, and more unbiased and global screening techniques that enable us to obtain data in throughput fashion such as DNA microarrays, to measure the relative expression of thousands of genes (Drigues et al., 2003). Another useful method is to estimate the

expression level of high numbers of genes by serial analysis of gene expression (SAGE), a technique based on sequencing of short sequence tags derived from tissue samples (Datson et al., 2001). Finally, subtractive hybridization (SH), which is based on constructing a subtracted cDNA library while screening differentially expressed genes (Alfonso et al., 2004a; b, see Materials and methods). Due to the strategy of the SH technique the final cDNA populations are enriched in those sequences that show differential expression which means that the respective mRNA is either up or downregulated by the experimental condition. Although these techniques are powerful tools to analyze the genes, expression results have to be confirmed by other techniques such as real-time polymerase chain reaction (RT-PCR), Northern blot or *in situ* hybridization. *In situ* hybridization provides the possibility to quantify mRNA and to localize the change in gene expression to specific neuronal subpopulation. However, it is much less sensitive than quantitative real-time PCR, requires more animals to investigate several genes, is time consuming and requires considerable technical efforts. Real-time PCR allows the determination of the mRNA levels of specific genes precisely, reliably and relatively fast. The use of a fluorescent dye is essential for detecting the PCR product formation. The increase in fluorescent emission can be read by a sequence detector in “real time”, during the course of the reaction and is a direct consequence of the target sequence amplification during the PCR reaction. The fluorescence values are plotted against the cycle number, giving the amplification plot. Then an arbitrary threshold (C_t) is chosen based on the variability of the baseline. This C_t can be used as a quantitative measurement of the input target sequence (see Materials and methods). However, it can only measure the mRNA transcripts in whole tissue samples. Using the present methodological approaches I was able to detect changes in gene expression, but whether the detected changes in their expression occur in serotonergic or non-serotonergic neurons has yet to be determined.

The SH technique has been used in the present thesis to identify genes in the DRN that are differentially expressed due to stress. Real-time PCR was employed to confirm the effect of stress and to investigate the effect of CIT treatment. To have an insight on the functional level (protein expression) of some of those differentially expressed genes, quantitative Western blot was performed, a valuable tool to

measure the relative abundance of the same protein in different populations of protein extracts. Therefore, the RNA isolation was performed using the Trizol® method, an organic solvent-based method, which gives the chance to precipitate RNA, DNA and protein stepwise in the same tissue sample. This provides the opportunity to analyze mRNA and protein in the same samples.

1.5 Aims of the thesis

The aims of the present thesis were to identify genes in the rat DRN that are regulated by chronic social stress and to investigate whether these genes are also regulated by treatment with the SSRI CIT. To achieve this aim, the work was performed as follows:

In the first part of the thesis, using a resident intruder paradigm, male Wistar rats were chronically stressed by daily social defeat during 5 weeks, and at the end of experiments brains were dissected. To find target genes that might be differentially regulated by chronic social stress in the DRN, I used subtractive cDNA hybridization. I purified mRNA from DRN of chronically stressed and control animals and generated two cDNA libraries containing sequences that were potentially regulated by the stress. From these libraries, I selected genes according to their functions for further quantitative analysis. Because stress is known to activate and increase 5-HT neurotransmission I determined expression of two genes involved in neurotransmission (synaptosomal associated protein-25 kD, SNAP-25, and synaptic vesicle protein 2b, SV2b) using real-time PCR. To investigate whether also glial cells might be affected by chronic social stress in the DRN, I also quantified mRNA for N-myc downstream-regulated gene 2 (NDRG2) that is expressed in astrocytes (Nichols, 2003). The gene for CREB binding protein (CBP) was also contained in the cDNA library after subtractive hybridization. Because CBP plays a role in activation of the transcription factor CREB that is known to be regulated by antidepressants and supposed to play role in psychopathologies (Nibuya et al., 1996; Dowlatshahi et al., 1998), I also analyzed expression of CBP mRNA. Expression of neuron-specific enolase (NSE), which is involved in cell energy metabolism and showed neurotrophic and neuroprotective effects *in vitro* (Hattori et al.,

1995) was also investigated. Genes directly related to 5-HT neurotransmission were not identified by subtractive cDNA hybridization, but quantitative real-time PCR was nevertheless performed for the serotonin transporter (SERT), the 5-HT_{1A} autoreceptor and tryptophan hydroxylase (TPH) genes 1 and 2.

The aim of the second part of the present thesis was to investigate the impact of chronic citalopram treatment (30 mg/kg/day, for 4 weeks administered via drinking water) on mRNA expression of the above genes in the DRN in stressed and naïve animals. The appropriate dose of citalopram was determined in a pilot study.

To analyze whether stress and citalopram also change protein expression in the DRN, Western blot experiments were performed. I analyzed the protein expression of NSE, SNAP-25, SV2b and TPH. To verify whether stress and CIT alter the expression of synaptic/synaptic vesicle proteins in general, I also analysed protein expression of syntaxin 1A and synaptophysin. Finally, to investigate whether the effects of stress and citalopram on gene expression are confined to the DRN, protein expression was also determined in the hippocampal formation, a limbic area known to be particularly sensitive to stress effects (Lopez et al 1998; 1999).

2 Materials and methods

2.1 *Experimental animals*

Experimentally naive male Wistar rats (Harlan-Winkelmann, Borcheln, Germany) weighing 180-200 g at the time of arrival were used for the study. They were housed individually in type III macrolon cages, with rat chow and water available ad libitum. The animal facility was maintained at 21°C with a reversed 12 h:12 h light/dark cycle (lights off at 10:00 am). After arrival, animals were habituated to the conditions for two weeks and handled daily (control phase). Lister Hooded male rats, weighing 300-350 g (Harlan-Winkelmann, Brochen, Germany) were used as residents. These rats were paired with sterilized females and housed in large plastic cages ($60 \times 40 \times 40$ cm = l $\times$ w $\times$ h) located in a separate room to the Wistar rats, but subjected to the same maintenance. All experimental manipulations were conducted during the dark phase of the light/dark cycle under dim red light. Animal experiments were conducted according to the European Council Directive of November 24, 1986 (86/609/ECC), and were approved by the Government of Lower Saxony, Germany. The animal experiments described in the present study were performed by Rafal Rygula (MSc).

2.2 *Social stress*

Chronic social stress was induced as described recently (Rygula et al., 2005). In the resident-intruder paradigm that took place under dim red light, the experimental (Wistar) rat was introduced into the home cage of an unfamiliar aggressive resident male. Before the start of the social defeat procedure, the female resident rat was removed from the resident's cages. Each experimental male Wistar rat was then transferred from its home cage to the resident's cage for one hour. Usually within 1-3 min, the intruder was attacked and defeated by the resident, as shown by freezing behaviour and submissive posture of the intruder, whereupon intruder and resident were separated. For the rest of the hour, the intruder was kept in a small wire-mesh compartment ($25 \times 15 \times 15$ cm) within the resident's cage to be protected

from direct physical contact but remaining in olfactory, visual and auditory contact with the resident. Thereafter, intruders were returned to their home cages. Two groups of animals *Stress* and *Control* were used for performing subtractive hybridization procedure and real-time PCR. Four groups *Control*, *Stress*, *Stress CIT* and *Control CIT*, were used for quantification of gene expression by real-time PCR and protein expression by Western blot (analysis of CIT effect, Table 1). Animals from *Stress* and/or *Stress CIT* groups were subjected to social defeat daily for five weeks. To avoid individual differences in intensity of the defeat, the intruders were confronted each day with a different resident. Control animals were handled daily throughout the entire experiment. Handling consisted of picking up each rat, transferring it to the experimental room and returning it to its home cage. Chronic CIT treatment was performed during the last four weeks of the experimental time course (Figure 1). Body weight was measured regularly at 12:00 am (two hours after lights off), first at the end of the control phase (baseline), then at weekly intervals (weeks 1, 2, 3, 4, and 5) during the entire treatment phase. Body weight gain was calculated as percentage of individual baseline body weight at the beginning of the experiment. At the end of the experiment, animals were decapitated, brains and adrenals were dissected and adrenal weights were measured and expressed as percentage of body weight.

Table 1. Experimental groups and number of animals per group

Experiment	Experimental groups			
	<i>Control</i>	<i>Stress</i>	<i>Stress CIT</i>	<i>Control CIT</i>
Subtractive cDNA hybridization	3	3	----	----
Quantitative real-time PCR and quantitative Western blotting	8	8	8	8

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Control	No Stress	No Stress				
Stress	No Stress	Stress				
Stress CIT	No Stress	Stress	Stress, Citalopram			
Control CIT	No Stress	No Stress	No Stress, Citalopram			

Figure 1. Design of the chronic social stress and chronic CIT treatment experiment. *Control* group was handled without being exposed to stress or drug. Animals from the *Stress* group were exposed to chronic social stress on a daily basis for five weeks and received no drug. The third group, *Stress CIT*, was stressed for five weeks and received CIT via drinking water starting after one week of stress and continuing for the following four weeks. In the last group, *Control CIT*, animals were only handled and chronically treated with CIT for the last four weeks. All animals were housed individually during the experimental time course. Number of animals per group=8.

2.3 Chronic administration of citalopram

CIT hydrochloride was kindly donated by Lundbeck (A/S, Copenhagen, Denmark). To attain a concentration of CIT in the blood of the treated rats in the range of a therapeutically effective CIT concentration in humans (30-130 ng/ml; Baumann et al., 2004), the dose of the drug was adjusted and monitored in a pilot study as we described before (Rygula et al., 2006). The drug was administered orally via drinking water as described (Montkowski et al., 1995). This route of administration was chosen to minimize stress effects that might be induced by injection and because in humans, antidepressants are in most cases applied orally. The amount of consumed water was measured daily by weighing the bottles. This procedure allowed us to adjust the dose of dissolved CIT to individual water consumption and body weight of each animal. Body weight was also controlled on a daily basis. Animals were treated chronically with CIT (30 mg/kg) for four weeks starting on week 3 (one week after the beginning of the social stress), while untreated animals received tap water. The drug dose was calculated and dissolved in the average volume of consumed water/rat/day (approx. 30 ml/day). The solution containing the drug

was freshly prepared every day and filled into opaque bottles. The bottles were weighed after preparation and 24 hours later to monitor drug intake. Fluid intake was monitored before and throughout the entire experiment, by weighing the bottles always at the same time of the day 12:00. At the end of the experiment, animals were decapitated and trunk blood of CIT treated animals was collected. Samples were stored in heparinized tubes. Plasma was stored at -20°C until assayed for drug and its metabolite (demethylcitalopram, DCIT).

2.4 Determination of citalopram and its metabolite

Analysis of CIT and its demethylated metabolite DCIT was kindly performed by Prof. C. Hiemke (University of Mainz). CIT was determined in blood serum using high performance liquid chromatography (HPLC) with column switching and ultraviolet (UV) spectroscopy by a method originally established for reboxetine (Hartter et al., 2000) and slightly modified. After high speed centrifugation (5 min at $10,000 \times g$) serum (0.1 ml) was injected into the HPLC system. On-line sample clean-up was performed on a column (10×4.0 mm i.d.) filled with silica CN material (20 μm particle sizes, MZ Analysentechnik, Mainz, Germany) using an eluent consisting of 8% (v/v) acetonitrile in deionized water to remove matrix constituents. The drug was eluted and separated on LiChrospher CN material (5 μm ; column size 250×4.6 mm i.d., MZ Analysentechnik) by acetonitrile-phosphate buffer (50:50; v/v, pH 6.4) at a flow rate of 1.5 ml/min and quantified by UV detection at 254 nm.

2.5 Dissection of brains

Animals were decapitated 24 hours after the last stress exposure. Brains were quickly removed, dissected into two parts by a cut perpendicular to the cortical surface along the caudal side of the hippocampus. The hippocampus was immediately dissected, frozen and kept at -80°C for later analysis. The caudal part of the brain was immediately frozen over liquid nitrogen and stored at -80°C until use. Tissue samples from the dorsal raphe nucleus were punched according to the method of Palkovits (1973). The caudal part of the brain was positioned on the specimen holder of a cryostat (at -18°C), and tissue was cut in caudal to rostral direction until -8.00 mm from Bregma (Paxinos and Watson, 1986; Figure 2). At this level, a pre-cooled sharpened hypodermic needle (inner diameter 1.3 mm, -20°C) was punched 1 mm deep into the DRN. This depth corresponds to the maximal caudo-rostral extension of the DRN. RNA was immediately isolated.

Figure 2. Schematic drawing of the anatomical level at which the DRN was punched out (grey area). DRD, nucleus raphe dorsalis part dorsalis; DRV, nucleus raphe dorsalis part ventralis. DRVL, nucleus raphe dorsalis part ventrolateralis. Adapted from Paxinos and Watson (1986). Scale bar = 1 mm.

2.6 Construction of subtractive cDNA library

2.6.1 Subtractive cDNA hybridization procedure

For generation of cDNA libraries, mRNA was purified from pooled DRN of 3 control and 3 stressed male Wistar rats and two subtracted cDNA libraries were constructed using the PCR-Select™ cDNA subtraction kit according to the manufacturer's manual (Clontech, Heidelberg, Germany). Briefly, double-stranded cDNA was generated from mRNA and subjected to restriction digestion with Rsa I. Tester and driver samples obtained from pooled DRN of *Stress* and *Control*, respectively, were used to generate the library enriched in genes upregulated by stress, and *vice versa* to generate the library with the stress downregulated genes (forward and reverse subtraction; Figure 3). Following digestion, the tester sample was split into two pools. Each pool was ligated with a different adaptor (N1 and N2R), denatured and hybridized with excess of denatured driver cDNA. The hybridization pools were mixed and a second round of hybridization was performed with an excess of denatured driver. The entire population of hybridization molecules was subjected to a primary and a second (nested) PCR to amplify the desired sequences. The amplified fragments were ligated into a PCR product cloning vectors. The ligated clones were transformed into *Escherichia coli* competent bacteria.

2.6.2 PCR analysis of subtraction efficiency

The efficiency of the subtraction procedure was evaluated according to the manufacturer's instructions using primers for the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The sequences of the GAPDH primers were as following: GAPDH 5'-primer: 5'-ACCACAGTCCATGCCATCAC-3' and GAPDH 3'-primer: 5'-TCCACCACCCTGTTGCTGTA-3'. This test is performed by comparing the abundance of known house keeping gene (GAPDH) before and after subtraction from both the forward and reverse libraries. The efficiency was tested as follows: Subtracted and unsubtracted samples were diluted 10 fold in water; all reagents required for PCR analysis were mixed in 0.5 ml tubes (Table 2). Tubes were incubated in a thermocycler using the following program: 94°C for 30 seconds, 60°C for 30 seconds and finally 68°C for 2 min for 18 cycles. Thereafter, 5 µl from

subtracted and unsubtracted samples were removed, and the subtracted samples were returned back into the thermal cycler for 25 cycles, thereafter 5 μ l from subtracted samples were removed. The 5 μ l samples were examined on an agarose (2%)/ethidium bromide gel.

Table 2. Reagents in the 0.5 ml tube for analysis of subtraction efficiency

Components	Volume (μ l)
Diluted subtracted or unsubtracted cDNA	1.0
GAPDH 3'-primer (10 μ M)	1.2
GAPDH 5'-primer (10 μ M)	1.2
Sterile water	22.4
10 $\times$ PCR reaction buffer	3.0
dNTP mix (10 mM)	0.6
50 $\times$ advantage cDNA polymerase mix	0.6

Figure 3. Schematic diagram of the subtractive cDNA hybridization procedure. The cDNA in which specific transcripts shall be detected is called “tester” and the reference cDNA is called “driver”. The hybridization procedure is performed twice, first with the “tester” and “driver”, and then a second time in the reverse manner with the original “tester” now becoming the “driver”, and the original “driver” becoming the “tester”. Type E molecules are formed only if the respective sequence is upregulated in the “tester” cDNA, and it is only this molecule which will be exponentially amplified by PCR.

2.7 Analysis of the cDNA clones

Clones with inserts ≥ 100 bp were sequenced using standard Hot Shot procedure (SEQLAB Sequence Laboratories GmbH, Göttingen, Germany). After sequencing, the non-vector sequences were used to search the public databases using the default databases search BLASTN (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the non-redundant nucleotide databases constructed from the Entrez databases (the National Centre for Biotechnology Information, NCBI). Prior to the BLASTN analysis, I removed the flanking nested primers keeping only the inserted sequences in order to avoid false BLASTN hits. Identified clones were compared among each other to detect and remove redundant clones. The remaining clones were compared to public databases for identification of the genes or the proteins represented by the cloned sequences.

2.8 Quantification of mRNA expression by real-time PCR

2.8.1 First-strand cDNA synthesis and real-time PCR procedure

To quantify mRNA isolated from the DRN, tissue dissected from individual animals was homogenized in Trizol[®] reagent (Invitrogen, Karlsruhe, Germany) for isolation of total RNA. DNase I digestion was performed and the total RNA was purified using phenol/chloroform/isoamylalcohol followed by isopropyl/sodium acetate precipitation. The integrity and quantity of the RNA was checked using RNA 6000 Nano LabChip[®] kit (Agilent Technologies, Böblingen, Germany). RNA was reverse-transcribed with Superscript II (Invitrogen, Karlsruhe, Germany). Briefly, first-strand cDNA was synthesized by adding the following components to a nuclease-free 0.5 ml tube: 1 μ l of Oligo(dT)₁₂₋₁₈ primers to 1 μ l dNTP mix (10 mM each), then 1 μ g total RNA from each sample was added, followed by adding sterile distilled water up to 12 μ l total reaction volume. Mixture was heated to 65°C for 5 min and quickly chilled on ice. Then 4 μ l of 5 $\times$ first-strand buffer, 2 μ l 0.1 M DTT and 1 μ l RNase OUT[™] (40 units/ μ l) were added into the tube. Contents were mixed gently and incubated at 42°C for 2 min in an air heater.

Finally 1 µl (200 units) of Superscript™ II RT was added, after gentle mixing tubes were incubated at 42°C for 1 hour. The reaction was then inactivated by heating the samples at 70°C for 10 min using thermomixer.

Primer sequences for target genes were designed using Primer Express software v2.0 (Application-based primer design software, Applied Biosystems, Darmstadt, Germany). Amplicons were 50-150 bp long. I selected the primers that amplify sequences close to the 3'-untranslated region (UTR) of the target genes (Table 3). Amplicons were amplified using 7500 Real-time PCR system (Applied Biosystems) with QuantiTect™ SYBR® Green PCR (Qiagen, Hilden, Germany). For components of the reaction mixture see Table 4. The real time cyclers conditions for the light cycler were used as follows: Initial PCR activation step lasting for 10 min at 95°C, followed by cycling steps; denaturation for 15 seconds at 95°C, annealing for 30 seconds at 60°C and extension 1 min at 72°C; these steps were repeated for 40 cycles. A heat dissociation protocol was used in order to verify that the SYBR green dye detected only one PCR product (Ririe et al., 1997). In general, the melting curve is an automated step performed by the software of the cycler, the protocol starts at 95°C for 15 second then temperature is decreased down to 65°C in certain rate, and then it rises back to 95°C. Then for the specific amplicon one single peak of maximal fluorescence will appear and then during heating phase back to 95°C the fluorescence signal will disappear, which corresponds to one specific product (according to the present protocol all our amplicons were around 50-150 bp long, maximum fluorescence occurred at 72-75°C). Any primer dimers or non-specific amplifications can be detected through their lower melting point.

Table 3. Primer sequences used for the quantitative real-time PCR

Gene name	Forward primer sequence	Reverse primer sequence
GAPDH	TGCCCCCATGTTTGTGATG	TGGTGGTGCAGGATGCATT
SV2b	CGTTCGCTGTTTCATGATGTGTT	AGAAGCAACTCCCAAAGAAGCC
CBP	TGTCCCGATAACGAGCTGATG	CTTGGAGATGCCCACAGAGTTC
NDRG2	GAGATGGTGGCCAGTGAAGAAC	AATGCCCCTGCTTCAATGTG
SNAP-25	CCATCTCCCTGT GGTTCGTCA	CAGCAATTTGGTTGTGCATAGC
NSE	ATAACTCTCTCCCCCAGCCCTA	GTGCAGCCGGAGAAAAAGACT
SERT	TCTGAAAAGCCCCACTGGACT	TAGGACCGTGTCTTCATCAGGC
5-HT _{1A}	GTCCTGCCTTTCTGTGAAAGCA	TATGGCACCCAACAACGCA
TPH2	TGAGAACCCCAAATCCTGCA	CCCAGCCAACAGACCTAACTGA
TPH1	AGCATAACCAGCGCCATGAA	GGCATCATTGACGACATCGAG

Table 4. Real-time PCR reaction components using QuantiTect™ SYBR green PCR kit

Components	Volume (µl/reaction)
RNase-free water	22
QuantiTect SYBR green PCR master mix	25
Forward primer	1
Reverse primer	1
cDNA	1

2.8.2 Data collection and analysis

Experiments were performed in triplicates for each cDNA sample. All samples were normalized against the house keeping gene GAPDH. The threshold cycle for each amplified sequence was determined and the percentage of the relative abundance of each amplified sequence to the amplified GAPDH sequence for each animal was calculated as described previously (Peirson et al., 2003). Relative quantification approach involves determining the change in gene expression relative to another set of experimental samples, in this case control group.

The simplest method for accurate relative quantification is by calculating the theoretical value R_0 . this based on the simple formula used to simulate a PCR up to the point of the plateau (Peirson et al., 2003):

$$X_n = X_0 \times (1+E)^n$$

X_n is the concentration of the template at cycle n , X_0 is the initial template concentration and E is the amplification efficiency. E has the value of 1.0 when exact amplification of the product occurs and 0 when the reaction fails. The basic concepts underlying real-time PCR include: First, the number of copies is proportional to accumulation of fluorescence (R). Second, E value approaches 1 when the efficiency is 100% (amplicons are as short as possible, which maximizes the efficiency). Therefore, the above equation can be reformulated as follows:

$$R_0 = R_{Ct} \times 2^{-Ct}$$

Ct is the threshold cycle and R_{Ct} is the fluorescence at this cycle (the actual threshold used for analysis). R_0 for each gene corresponds to the number of template copies in each sample. Since I have started cDNA synthesis using the same RNA amount for each sample then by calculating R_0 for GAPDH (house keeping gene), from our four experimental groups one can figure out whether GAPDH expression significantly varies among four groups, and so if it is suitable as house keeping gene to normalize the final data. For normalizing the data R_0 for each gene is calculated along with R_0 for GAPDH from each sample. The level of expression of a candidate gene (e.g. A) will be calculated as the percentage of its R_0 to the GAPDH R_0 according to the following equation:

$$\% \text{ of GAPDH} = R_{0A} \times 100 / R_{0\text{ GAPDH}}$$

2.9 Quantitative immunoblotting

Total protein extracts from the DRN and/or hippocampus were obtained from the phenolic phase of the pooled Trizol homogenates after RNA isolation according to the manufacturer's instructions. Samples from each group were pooled and proteins were precipitated with isopropyl alcohol and then washed with 0.3 M guanidine hydrochloride in 95% ethanol. The pellet was dissolved in 1% SDS and protein concentration was measured using modified Lowry method (Stoscheck, 1990). This modified method, which outdated the 2-steps Lowry method, performed only in one step at room temperature and the colour of the reaction is more stable. The method is compatible with salt solutions, provides more linear response and is less likely to become saturated, compared to the original Lowry method. For performing this assay procedure the protein samples are diluted (400 µl total volume per dilution) to an estimated protein concentration of 0.025-0.25 mg/ml with the dissolving buffer (in this case 1% SDS), reference and standards samples are prepared as well (final volume 400 µl), then 400 µl of 2× Lowry concentrate were added, which consists of copper reagent (0.6 M sodium carbonate, 5.3 mM cupric sulphate and 2.4 mM potassium tartrate), 1 M sodium hydroxide and 1% SDS solution (3:1:1 ratio). Tubes were mixed thoroughly and incubated at room temperature for 10 min. Then 200 µl of 0.2 N Folin reagent were added, tubes were vortexed immediately and incubated at room temperature for 30 min. The absorbance was read at 595 nm. A standard curve was generated and the concentration of samples was estimated from the curve. Each sample was determined in duplicate. The average of the absorbance was used to estimate the concentration. All samples were handled simultaneously.

The concentrations of total protein loaded on the gel were adjusted so that the respective proteins (NSE, SNAP-25, SV2b, TPH, syntaxin 1A and synaptophysin) yielded a clearly visible band. For analysis of SNAP-25, 0.3 µg total proteins from DRN of stressed and control animals, respectively, were boiled in Laemmli buffer, loaded and separated on 12% SDS-PAGE. After electrophoresis, protein was transferred to nitrocellulose membrane from the gel. The membrane was blocked with 5% BSA in TBS (10 mM Tris, 150 mM NaCl, pH 7.6) overnight at 4°C. Following blocking, the membrane was washed with TBS-T (TBS, 0.05% Tween 20), then incubated with the primary anti-SNAP-25 antibody (dilution

1:10000, Synaptic Systems, Göttingen, Germany) for 2 hours at room temperature. Thereafter, the membrane was washed with TBS-T and incubated with peroxidase-labeled anti-mouse secondary antibody (dilution 1:3000, Santa Cruz Biotechnology, Inc., California, USA) for 1 hour at room temperature. After further washing, the membrane was subjected to enhanced chemiluminescence amplification (ECL) and the bands were visualized on Hyperfilm™ ECL films according to the manufacturer's manual (Amersham Bioscience Ltd, UK). With NSE analysis, 5 µg of total proteins were separated on 10% SDS-PAGE gel. After blotting, the membrane was incubated with anti-NSE primary antibody (dilution 1:500, Chemicon International, Inc., Canada) followed by peroxidase-labeled anti-rabbit secondary antibody (dilution 1:7500, DAKO A/S, Denmark). For SV2b, 30 µg of total protein were separated on a 10% SDS-PAGE gel. After blotting, the membrane was incubated with anti-SV2b primary antibody (dilution 1:1000, Synaptic Systems) followed by peroxidase-labeled anti-rabbit secondary antibody (dilution 1:7500, DakoCytomation, Denmark). For TPH detection 60 µg total protein were loaded in each lane. After blotting, membrane was incubated with anti-TPH primary antibody (dilution 1:100, Abcam Ltd, Cambridge, UK), then with peroxidase-labeled anti-goat secondary antibody (dilution 1:1000, DAKO A/S, Denmark). For syntaxin 1A detection 6 µg total protein were loaded for each group. Anti-syntaxin 1A primary antibody was used (dilution 1:1000, Synaptic Systems) followed by peroxidase-labeled anti-mouse secondary antibody (dilution 1:3000, Santa Cruz Biotechnology, Inc.). Finally, for synaptophysin detection, 30 µg total protein were loaded per lane and anti-synaptophysin primary antibody was used at dilution 1:10000 (Synaptic Systems) followed by peroxidase-labeled anti-mouse secondary antibody (dilution 1:3000, Santa Cruz Biotechnology, Inc.). The simultaneous detection of β-actin, with mouse monoclonal anti-β-actin antibody (dilution 1:2000, Sigma, Taufkirchen, Germany), served as internal loading control and was used for normalization of the optical density of the bands. The relative optical density of bands on the ECL films was quantified using the AIS40 imaging software (Imaging System, Inc., St. Catherines, Canada). Relative optical density (ROD) of bands was calculated as the ratio of the OD of the respective protein band to the internal control's OD band. Data

were presented as percentage of the mean ROD from the control group. Data for each protein were obtained from 3 independent experiments.

2.10 Statistics

All data are tested for normality (95% confidence interval). Results are presented as mean $\pm$ SEM. A probability level of 95% was chosen to determine statistical significance ($p < 0.05$). Statistical analysis was performed with Graph Pad Prism 4.0 (GraphPad Software, Inc., San Diego, USA). Statistical analyses and comparisons were performed using two-tailed unpaired Student's *t*-test, one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test for multiple comparisons and two factorial (stress *vs* treatment) ANOVA followed by Bonferroni *post hoc* test.

3 Results

3.1 *Chronic stress-induced gene expression in the DRN*

3.1.1 **Subtractive cDNA libraries enriched in stress regulated genes**

To identify genes potentially involved in stress-induced activation of DRN neurons subtractive cDNA hybridization was performed. Two cDNA libraries from pooled tissue of the DRN of chronically stressed rats and controls were generated and 247 clones (the average insert length ~200 bp) were sequenced from the libraries (forward and reverse). Sequences were compared against public databases both at the DNA and the protein level. It was found that 68% of the obtained sequences codify known proteins or genes, 2% encode ESTs published in at least one database, 13% are novel sequences matching only human or mouse genome, and 17% are novel sequences that do not match with any public database. The sequences matching known proteins or genes (168 clones) were compared against each other for redundancy, and it was found that they matched 36 genes/proteins. Assigning the known genes to functional groups resulted in the following categories: Extracellular matrix proteins, proteins related to ion transport, cell energy/metabolism, protein synthesis and degradation, signal transduction including kinases, synaptic and other proteins related to neurotransmission, transcription factors, and RNA and DNA binding proteins. Genes that could not be classified were included as miscellaneous (Table 5).

Table 5. Identified sequences cloned after subtractive cDNA hybridization (libraries with genes potentially up or downregulated by chronic social stress). Sequences were grouped according to functional aspects

Accession number	Gene name
<i>Extracellular matrix:</i>	
Upregulated genes:	
XP_342327	Procollagen, type XI, alpha 1
NM_031583	Chondroitin sulfate proteoglycan 6
NM_012656	Secreted acidic cysteine rich glycoprotein, osteonectin (SPARC)
<i>Ions concentration:</i>	
Upregulated genes:	
NM_134363	Solute carrier family 12, member 5 (potassium/chloride transporter)
Downregulated genes:	
NM_017290	ATPase, Ca ⁺² transporting, cardiac muscle
<i>Energy:</i>	
Upregulated genes:	
M64496	Cytochrome c oxidase polypeptide II
P00159, J01436	Cytochrome b
NM_145783	Cytochrome c Va-subunit
AAN77603	NADH dehydrogenase subunit 4
NM_139325	Neuron-specific enolase (NSE)
<i>Protein synthesis and/or degradation:</i>	
Upregulated genes:	
XM_221325	Translation initiation factor 4A, isoform 2, eIF-4AII
NM_031978	26S proteasome, subunit p112 (PSMD1)
NM_031106, BC059132	Ribosomal protein L37
NM_012876	Ribosomal protein S29
NM_031706, XM_237702	40S Ribosomal protein S8
Downregulated genes:	
NM_031108	Ribosomal protein S9
<i>Signal transduction and kinases:</i>	
Upregulated genes:	
NM_133583.	N-myc downstream-regulated gene 2 (NDRG2)
U36444	PCTAIRE-1 kinase
XM_341764	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform (Ppp2r1a)

Table 5 continues on the following page

Downregulated genes:

NM_053723

LanC-like 1 lanthionin synthase

Synaptic proteins and neurotransmission:

Upregulated genes:

NM_030991

Synaptosomal associated protein-25 (SNAP-25)

NM_057207, AF372834

Synaptic vesicle glycoprotein protein 2b (SV2b)

X70496, Q08326

GDP-releasing protein; guanine nucleotide releasing protein;
Mss4 protein (guanine nucleotide exchange factor MSS4,
RAB interacting factor)*Transcription regulation:*

Upregulated genes:

AY462245

CREB binding protein (CBP)

A23692

CCAAT-binding, chain A1

RNA and DNA binding:

Upregulated genes:

XM_223190

Heterogeneous nuclear ribonucleoprotein D-like; A+U-rich
element RNA binding factor

XM_216420

DNA polymerase epsilon p17 subunit

Miscellaneous:

Upregulated genes:

XM_217436

DanJ homologue (Hsp 40) superfamily B member 2,
neuronal DANJ like 1

NM_133544

Diabetes associated protein in insulin sensitive tissue

AF439750

Myelin basic protein

NM_013015

Brain prostaglandin D2 synthase

XM_340768

RIKEN cDNA 0610007p22 similar to hypothetical protein.

XM_225699

Similar to hypothetical protein (LOC307249)

XM_486647

Dedicator of cytokinesis 11 (Dock11)

Q63199

Tumor necrosis factor receptor superfamily member 6 precursor

Downregulated genes:

XM_214061

Ab2-450 T-cell activation protein

3.1.2 GAPDH gene transcripts: efficiency of cDNA subtraction

No sequences complementary to GAPDH gene were detected in the cDNA libraries after SH. To test whether GAPDH gene transcripts were really removed by the hybridization, the PCR-based subtraction efficiency test was performed using primers for GAPDH gene. No GAPDH band was detected after 18 or 25 cycles in the forward and reverse subtraction samples (Figure 4A), while in unsubtracted sample a clear band was observed at approx. 600 bp after only 18 cycles (Figure 4B). Beside that, the expression of GAPDH from the different groups of animals did not vary significantly; indicating that the house keeping gene GAPDH is suitable for normalizing the real-time PCR data.

Figure 4. PCR-based subtraction efficiency test. A. Note that no bands are visible in the forward (1) and reverse (2) subtracted samples, after 18 and 25 cycles. B. In contrast in the unsubtracted sample, a clear band at 600bp, which is the amplified GAPDH sequence, was detected after only 18 cycles (arrow).

3.1.3 Stress-upregulated genes

From the cDNA libraries with sequences potentially up or downregulated by the chronic stress I selected 5 genes according to their functions: 2 related to synaptic mechanisms, SNAP-25 and SV2b, the astrocytic gene NDRG2, CBP, and NSE that is involved in cell energy metabolism and showed neurotrophic and neuroprotective effects in vitro. We analyzed expression of these genes by quantitative real-time PCR. The mRNA levels for the following genes were found to be increased in the DRN of the Wistar rats after 5 weeks of daily social defeat (Figure 5): SNAP-25 (by 35%), SV2b (by 67%), CBP (by 49%), NSE (by 45%) and NDRG2 (by 31%). Statistical analysis using *t*-test showed significant effects of stress on mRNA expression for 4 genes: SNAP-25 ($t_{(14)}=3.09$, $p<0.01$), CBP ($t_{(14)}=2.76$, $p<0.05$), NSE ($t_{(14)}=3.01$, $p<0.01$) and NDRG2 ($t_{(14)}=2.35$, $p<0.05$). SV2b showed a trend towards reduced expression in stressed animals ($t_{(14)}=2.13$, $p=0.051$, Figure 5).

3.1.4 Genes related to serotonin neurotransmission

Sequences of genes related to 5-HT neurotransmission were not contained in the cDNA libraries after SH. Nevertheless it was tested by quantitative real time PCR whether stress affects mRNA expression of those genes. Expression of genes directly related to 5-HT neurotransmission, 5-HT_{1A}, TPH2 and SERT mRNA, was not significantly changed by the chronic social stress (Figure 6). However, chronic social stress induced a significant increase in TPH1 mRNA expression (more than 3.5 folds, $t_{(14)}=5.85$, $p<0.001$, Figure 6).

Figure 5. Effect of chronic social stress on expression of genes in the DRN: Synaptosomal associated protein-25 kD (SNAP-25), synaptic vesicle glycoprotein 2b (SV2b), CREB binding protein (CBP), N-myc down-stream regulated gene 2 (NDRG2) and neuron-specific enolase (NSE). Data were generated with real-time PCR. Transcript expression is presented as percentage of mRNA for the housekeeping gene GAPDH. Significant differences between stressed and control animals are indicated by asterisks (n=8/group; two-tailed unpaired Student's *t*-test; *, $p < 0.05$; **, $p < 0.01$). SV2b showed a trend towards increased expression in stressed animals ($p = 0.051$). Data are expressed as mean $\pm$ SEM.

Figure 6. Expression of serotonin related genes: Serotonin transporter (SERT), 5-HT_{1A} autoreceptor (5-HT_{1A}) and tryptophan hydroxylase 1 and 2 (TPH1 and 2) after chronic social stress. Data were generated by real-time PCR. Transcript expression is presented as percentage of mRNA for the housekeeping gene GAPDH (n=8/group). Except for TPH1 mRNA, no significant differences were found. Significant differences between stressed and control animals are indicated by asterisks (n=8/group; two-tailed unpaired Student's *t*-test; ***, *p*<0.001). Data are expressed as mean $\pm$ SEM.

3.2 Citalopram treatment and effects on stress-regulated genes

3.2.1 Drug monitoring of citalopram

Four weeks of CIT treatment resulted in the following concentrations of the parent compound and its metabolite DCIT in the blood of all treated rats: CIT, 108.1 ± 15.94 ng/ml and DCIT, 335.7 ± 41.89 ng/ml (16 animals).

3.2.2 Effect of citalopram on stress-upregulated genes

CIT treatment significantly blocked the stress-induced upregulation of three genes in the *Stress CIT* group, SV2b ($p < 0.01$), CBP ($p < 0.001$) and NDRG2 ($p < 0.001$). In contrast, CIT had no significant effect on expression of mRNA for these genes in the *Control CIT* group (Figure 7). Chronic CIT significantly upregulated mRNA for in *Control CIT* (difference to *Control*, $p < 0.01$). CIT also increased mRNA in the *Stress CIT* group, however, the difference between *Stress* and *Stress CIT* was not significant. SNAP-25 mRNA expression did not significantly vary among the four experimental groups (Figure 7). To analyze the effect of stress, drug treatment and the stress $\times$ drug treatment interaction a two-way ANOVA (Table 6) followed by Bonferroni *post hoc* test was performed. The *post hoc* test did not show significance in the stress-induced upregulation of the NSE and SNAP-25 mRNA. However, comparing only *Stress* and *Control* groups using two-tailed unpaired Student's *t*-test revealed significant effects of stress as follows: NSE $p < 0.01$, and SNAP-25 $p < 0.05$.

Figure 7. Chronic CIT treatment reversed the stress-induced mRNA upregulation of the following genes: Synaptic vesicle glycoprotein 2b (SV2b), CREB binding protein (CBP) and N-myc down-stream regulated gene 2 (NDRG2). CIT showed a stress-independent upregulation of the mRNA of neuron-specific enolase (NSE) in both treated groups, *Stress CIT* and *Control CIT*. The difference between groups in the expression of synaptosomal associated protein-25 kD (SNAP-25) mRNA was not significant. Data were generated with real-time PCR. Transcripts expression was normalized to the GAPDH housekeeping gene and presented as percentage of GAPDH mRNA. Significant differences between *Stress* and *Control CIT* vs *Control* are indicated by *asterisks* (two-way ANOVA followed by Bonferroni *post hoc* test; *, $p < 0.05$; **, $p < 0.01$). The significant differences between *Stress* and *Stress CIT* are indicated by *plus signs* (two-way ANOVA followed by Bonferroni *post hoc* test; ++, $p < 0.01$; +++, $p < 0.001$). Data are expressed as mean $\pm$ SEM.

3.2.3 Effect of citalopram on genes related to serotonin neurotransmission

The impact of chronic CIT treatment on selected genes related to serotonin neurotransmission was explored: SERT, 5-HT_{1A}, TPH2 and TPH1. Statistical analysis revealed significant downregulation in 5-HT_{1A} mRNA in the *Stress CIT* group in comparison to *Stress* ($p < 0.01$, Figure 8). CIT significantly reduced TPH2 mRNA in all treated animals independently from the stress factor as follows: *Stress CIT* group ($p < 0.001$) and *Control CIT* ($p < 0.01$, Figure 8). CIT significantly upregulated TPH1 mRNA in *Control CIT* ($p < 0.001$, Figure 8). CIT significantly reversed the stress-induced upregulation of TPH1 mRNA in the *Stress CIT* in comparison to *Stress* ($p < 0.001$). Two-way ANOVA yielded effects of the drug treatment and stress $\times$ drug interactions (Table 6). CIT had no significant effect on the SERT mRNA expression (Figure 8).

Table 6. Two-way ANOVA analysis of real-time PCR data

Gene	Stress effect	Drug effect (CIT)	Stress $\times$ Drug interaction
SV2b	$F_{(1)}=5.32, p<0.05$	$F_{(1)}=5.27, p<0.05$	$F_{(1)}=5.13, p<0.05$
CBP	$F_{(1)}=3.94, p=0.057$	$F_{(1)}=21.91, p<0.0001$	$F_{(1)}=11.20, p<0.01$
NDRG2	$F_{(1)}=5.47, p<0.05$	$F_{(1)}=22.08, p<0.0001$	$F_{(1)}=4.43, p<0.05$
NSE	ns	$F_{(1)}=17.27, p<0.001$	ns
SNAP-25	ns	ns	$F_{(1)}=4.73, p<0.05$
SERT	ns	ns	ns
5-HT _{1A}	ns	$F_{(1)}=7.03, p<0.05$	$F_{(1)}=5.79, p<0.05$
TPH2	ns	$F_{(1)}=25.84, p<0.0001$	ns
TPH1	ns	ns	$F_{(1)}=46.00, p<0.0001$

ns: not significant

Figure 8. Effects of the chronic CIT treatment on mRNA expression of serotonin related genes in the DRN of stressed and control animals: Serotonin transporter (SERT), somatodendritic 5-HT_{1A} receptor (5-HT_{1A}) and tryptophan hydroxylase 1 and 2 (TPH2 and TPH1). Data were generated with real-time PCR. Transcripts expression was normalized to the GAPDH housekeeping gene and presented as percentage of GAPDH mRNA. Significant differences between *Stress* and *Control CIT* vs *Control* are indicated by asterisks (two-way ANOVA followed by Bonferroni *post hoc* test; **, $p < 0.01$; ***, $p < 0.001$) while the significant difference between *Stress* and *Stress CIT* is indicated by plus signs (++, $p < 0.01$; +++, $p < 0.001$). Expression of SERT mRNA did not differ between groups. Data are expressed as mean $\pm$ SEM.

3.3 Western blot analysis of proteins in the DRN

Expression of NSE, SNAP-25, SV2b, TPH, syntaxin 1A and synaptophysin proteins was quantified by Western blot analysis. The blotting membrane revealed one clear band for NSE in the molecular range of 58 kD (Figure 9A). Chronic stress increased the optical density of this band by approx. 2 fold ($p < 0.01$). Chronic CIT treatment increased the NSE protein expression in *Control CIT* ($p < 0.05$) and had no effect in the *Stress CIT* group ($p < 0.05$, Figure 9A). For SNAP-25, a band in the molecular weight range of 25 kD was detected (Figure 9B), and chronic stress increased the intensity of this band in the *Stress* group (by 23%). Although there was a trend towards normalization in the *Stress CIT* group, one-way ANOVA revealed no significant changes among groups. However, comparing only *Stress* and *Control* groups using two-tailed unpaired Student's *t*-test revealed a significant difference ($p < 0.05$). For SV2b, a strong band of the expected molecular weight (66 kD) was detected showing the common electrophoretic pattern of the glycoprotein SV2b. The optical density of the 66 kD band was significantly increased after stress (by 64%; $p < 0.05$; Figure 9C). CIT reversed the stress-induced upregulation of SV2b protein specifically in *Stress CIT* group ($p < 0.05$, in comparison to *Stress* (Figure 9C). No effect of CIT on SV2b expression was observed in the *Control CIT* group. Chronic social stress increased the TPH protein (55 kD) band (by 38%, $p < 0.001$; Figure 9D). Chronic CIT treatment significantly blocked the stress-induced TPH protein upregulation in the *Stress CIT* group ($p < 0.001$, in comparison to *Stress*; Figure 9D). No effect of CIT on the intensity of the TPH band was observed in *Control CIT* animals. Stress and/or CIT treatments had no effect on the bands representing syntaxin 1A and synaptophysin (Figure 10).

Figure 9. Protein expression in the DRN. The blotted membranes are shown on the left and quantification of the relative optical density (ROD) of the bands is shown on the right. Western blot analysis revealed that chronic social stress increased expression of neuron specific enolase (NSE, A), synaptosomal associated protein-25 kD (SNAP-25, B), synaptic-vesicle glycoprotein 2b (SV2b, C) and tryptophan hydroxylase (TPH, D). Equal amounts of proteins were loaded in each lane of the respective gel. The simultaneous detection of β -actin served as internal loading control and for normalization of the data. The ROD of bands is presented as percentage of the mean data from the *Control* group. Data were analyzed using one-way ANOVA followed by Bonferroni *post hoc* test for multiple comparisons. Significant differences to *Control* are indicated by asterisks (*, $p < 0.05$ and **, $p < 0.01$) and the significant difference between *Stress* vs *Stress CIT* groups is indicated by plus (+, $p < 0.05$ and ++, $p < 0.01$). Results were obtained from three independent experiments and presented as mean $\pm$ SEM.

Figure 10. Protein expression in the DRN. Representative blotted membranes are shown. Western blot analysis revealed that neither stress nor CIT caused changes in expression of syntaxin 1A (A) and synaptophysin (B). The simultaneous detection of β -actin served as internal loading control. Three independent experiments were performed.

3.4 Western blot analysis of hippocampal proteins

One-way ANOVA followed by Bonferroni *post hoc* test revealed that chronic social stress induced a significant upregulation of the NSE protein ($p < 0.05$, Figure 11A). The CIT treatment had no effect in both treated groups, neither *Stress CIT* nor *Control CIT*. With regard to synaptic proteins SNAP-25 and syntaxin 1A, chronic stress had no effects on SNAP-25 protein, while CIT upregulated SNAP-25 in the *Control CIT* group ($p < 0.05$, Figure 11B). The CIT treatment slightly reduced the SNAP-25 protein in the *Stress CIT* group but the effect was not significant. In contrast, chronic stress upregulated syntaxin 1A protein ($p < 0.01$, Figure 11C). CIT reversed the stress effect in the *Stress CIT* group ($p < 0.05$ in comparison to *Stress*), and CIT slightly reduced syntaxin 1A in *Control CIT* but this effect was not significant. Results obtained for SV2b and synaptophysin were excluded from the present thesis because the patterns of the bands varied from experiment to experiment.

Figure 11. Protein expression in the hippocampal formation. The blotted membranes are shown on the left and quantification of the relative optical density (ROD) of the bands is shown on the right. Western blot analysis revealed that chronic social stress increased protein expression of neuron specific enolase (NSE, A) and syntaxin 1A (C), but had no effect on synaptosomal associated protein-25 kD (SNAP-25, B). CIT reversed the stress effect on syntaxin 1A protein expression, but had no effect on NSE. CIT increased the SNAP-25 expression in the *Control CIT* group. Equal amounts of protein were loaded in each lane of the gel. The simultaneous detection of β -actin served as internal loading control and for normalization of the data. The ROD of bands is presented as percentage of the mean of the data from the *Control*. Data were analyzed using one-way ANOVA analysis followed by Bonferroni *post hoc* test for multiple comparisons. Significant differences to *Control* are indicated by *asterisks* (*, $p < 0.05$ and **, $p < 0.01$) and the significant differences to *Stress* are indicated by *plus* (+, $p < 0.05$ and ++, $p < 0.01$). Data are presented as mean $\pm$ SEM.

3.5 Body and adrenal weight

Both chronic stress and CIT resulted in reliable effects on body weight gain. Stressed rats gained less body weight than *Control* rats after 2 ($p<0.05$), 3 ($p<0.001$), 4 ($p<0.001$) and 5 weeks ($p<0.001$) of social stress (Figure 12). CIT caused a significant reduction in body weight gain after 2 ($p<0.001$), 3 ($p<0.001$) and 4 ($p<0.001$) weeks of treatment (corresponding to experimental weeks 3, 4 and 5, respectively). Two-way ANOVA revealed a significant effect of the stress ($F_{(3)}=60.32$, $p<0.0001$), a significant effect of CIT ($F_{(4)}=319.16$, $p<0.0001$) and stress $\times$ time interaction ($F_{(12)}=3.56$, $p=0.0001$; Figure 12).

Adrenal weight (expressed as percentage of body weight) was significantly increased in stressed animals. Two-way ANOVA showed a significant effect of stress only ($F_{(1,0)}=11.38$, $p<0.01$), *post hoc* test showed a significant difference between the *Stress* (0.01509 ± 0.00059) and the *Control* group (0.01215 ± 0.00105 , $p<0.05$). CIT had no effect on stress-induced adrenal hypertrophy in the *Stress CIT* animals (0.01536 ± 0.00090). No change in adrenal weight was observed in the *Control CIT* group (0.01254 ± 0.00079).

Figure 12. Effects of chronic social stress and CIT on body weight gain. Body weight gain was expressed as percentage of the initial body weight, and measured at 1-week intervals. Data are presented as mean $\pm$ SEM ($n=8$ per group).

4 Discussion

The present thesis provides insight into gene regulation within the DRN neural network in response to chronic social stress and a treatment with the SSRI citalopram. SNAP-25 and SV2b, but no other proteins involved in synaptic neurotransmission were found to be upregulated after stress at the mRNA and protein level. The chronic treatment with the SSRI CIT reversed the stress-induced upregulation of SV2b.

Stress is known to stimulate 5-HT neurotransmission and the present data indicate potential molecular mechanisms that may underlie increased 5-HT activity. Consistent with the view that stress activates 5-HT neurons, it was found that expression of the 5-HT biosynthesis enzyme TPH was upregulated after stress, apparently due to upregulation of mRNA for the TPH1 gene. CIT normalized the stress effect on TPH protein expression but displayed a dual pattern of action on expression of mRNA for the two distinct TPH genes, 1 and 2. In addition, one of the elements known to regulate 5-HT release was also found to be affected by CIT under stress conditions, namely the SSRI reduced 5-HT_{1A} mRNA. Furthermore, the present findings provide evidence for the involvement of genes related to neuroplasticity (CBP), neuronal survival (NSE) and glia-mediated plasticity (NDRG2) in stress-induced processes in the DRN. CBP and NDRG2 were upregulated by chronic stress, and CIT reversed the stress effects specifically in the *Stress CIT* group. Expression of NSE displayed a distinct regulatory pattern in that mRNA and protein were upregulated by both chronic stress and CIT. Also in the hippocampal formation, NSE expression was modulated by stress. However, CIT action on this protein was found to be specific to the DRN indicating that the regulatory effect of CIT is region specific. The effects of stress/CIT on expression of proteins related to synaptic functions in the hippocampus differed from those in the DRN.

4.1 *Methodological aspects*

4.1.1 Subtractive cDNA hybridization

To identify genes that are regulated by stress, an unbiased approach based on subtractive cDNA hybridization (SH) was adopted in the present study. This method allows comparing two populations of mRNA in that cDNA clones that are obtained with this technique correspond to transcripts that are enriched in one population compared with another population of mRNA molecules. The basic theory behind cDNA subtraction is that the tester and the driver cDNA are hybridized twice, and the hybrid sequences are then removed (see Materials and methods). Consequently, the remaining unhybridized cDNA sequences represent genes that are present in the tester but absent in the driver cDNA. Following PCR amplification steps, the differentially expressed cDNA transcripts are ligated into a PCR-product cloning vector. This ligation is a competitive process that depends primarily on the abundance of a certain transcript in the cDNA populations and therefore, rare messages may not be obtained. Mathematical calculations have revealed that an effective enrichment of a target transcript is determined by its concentration ratio between tester and driver, rather than its absolute concentration. It has been therefore, concluded that SH cannot exclude all non-differentially expressed genes from the libraries, and low-abundant transcripts are difficult to detect (Duguid and Dinauer, 1990; Hara et al., 1991; Ji et al., 2002). Based on those considerations it is possible that in the present thesis, the differential expression of low abundant genes such as genes related to 5-HT neurotransmission, G-protein coupled receptors and cytokines was not detected. In particular, transcripts of genes directly related to 5-HT biosynthesis were not contained in the cDNA libraries although it turned out that TPH1 mRNA was significantly upregulated by the chronic stress as determined by quantitative real-time PCR. Based on the quantitative real-time PCR data the relative abundance of TPH1 transcripts in the mRNA population is 10 times less than the least abundant gene (e.g. CBP). This means that TPH1 is expressed at a low level and is consequently hard to detect SH. However, on the basis of the other changes in gene expression observed here one may assume that a 30% change is enough to detect the respective transcripts after subtractive hybridization if they are of relatively high abundance.

SH does have an advantage over cDNA microarray screening techniques because the latter are biased due to the fact that they can only detect differential expression of those genes that are present on the chip. Using SH, libraries of clones with putatively up and downregulated sequences were generated after chronic stress from the DRN. In total, 1703 clones were obtained, those containing inserts ≤ 100 bp were excluded, and 554 clones were stored. Clones were sequenced, compared against each other to remove redundant clones, and compared to databases. It was found that they match known and yet unknown genes. Out of those sequences, 247 sequences matched known genes (approx. 1-4 clones per gene), then they were categorized into 8 groups representing probably the cellular functions affected by chronic stress (see Table 5). Some putatively upregulated genes were selected based on their functions, and their mRNA expression was quantified by real-time PCR to validate the SH libraries and to substantiate their regulation by stress.

4.1.2 Quantitative real-time PCR

In the present thesis quantitative real-time PCR has been used to determine mRNA expression of genes putatively regulated by chronic stress and CIT. Furthermore, expression of sequences directly related to 5-HT neurotransmission, though not contained in the cDNA libraries, was also quantified.

Quantitative real-time PCR is the method of choice used to quantify mRNA expression of genes whose differential expression occurs at a very low level. It is regarded as a sensitive and accurate method (Wang and Brown, 1999) that is suitable to confirm the differential expression of genes obtained by screening techniques such as SH. The use of a fluorescent dye is essential for detecting the PCR product formation. Among different reporter systems such as molecular beacon, scorpion primers and SYBR green (see Giulietti et al., 2001) the latter system is very useful to quantify the expression of many genes, avoiding the use of other more expensive systems. The other reporter systems require special considerations for each gene although they may be more specific than SYBR green. This specificity problem arises due to the fact that SYBR green is a DNA-binding dye that incorporates into double stranded DNA. It emits no fluorescence in its free form, but once bound to double stranded DNA

it emits fluorescence. Because SYBR green binds to any double stranded DNA it may not only detect the specific target, but also non-specific PCR products and dimers of the primer. To avoid detection of false double stranded molecules, melting curve should be generated (Ririe et al., 1997, see Materials and methods), primers should be carefully designed and reaction conditions should be optimized, potentially by using Hot Start PCR (see Materials and methods). Indeed, all those considerations have been undertaken while performing the quantitative real-time PCR experiments in the present thesis.

4.1.3 Quantitative Western blot

Protein blotting is the transfer of proteins separated by electrophoresis from the gel to a membrane. When the protein is bound to the membrane it is accessible for different detection methods (Towbin et al. 1979; Renart et al., 1979; Gershoni and Palade, 1983). Among these, the immunoblot is widely used because it not only detects a protein but provides also insight into its size (Renart et al., 1979). Moreover, when coupled with densitometry, immunoblots can be used to quantify proteins and compare their abundance in different samples. In order to obtain reproducible and reliable results some considerations should be undertaken. Among different factors that may affect protein quantification in the blot is the amount of total protein loaded on the SDS polyacrylamide gel. Of course, the optical density of the band increases in relation to the amount of loaded protein, however, from a certain point onwards, the concentration vs. optical density curve reaches a plateau so that any change in the protein concentration within a respective band cannot be reliably determined. On the other hand, for each protein special conditions of electrophoresis such as concentrations of reducing agents in the loading buffer, the acrylamide concentration in the gel and electroblotting conditions have to be optimized. Also the optimal dilution of primary and secondary antibodies is a crucial issue to avoid nonspecific binding and high background. In the present thesis, the respective control experiments were performed prior to the determination of the amount of protein in the bands. In addition, an internal loading control, in this case β -actin, that allows correcting for differences in amounts of total protein loaded in each lane of a gel was also included in each Western blot.

In the present thesis, quantitative Western blotting was used to quantify the expression of selected proteins. In order to obtain consistent reproducible results the above mentioned considerations were taken into account. However, it is important to note that difficulties such as non-detectable bands, non-specific binding, strong background, smear-like patterns are often experienced with Western blot. Beside that, other factors related to the protein characteristics may interfere with mastering this technique. Those difficulties begin with the protein extraction procedure, for instance membrane embedded proteins/glycoproteins (e.g. SV2b, synaptophysin) are more lipophilic than others, which makes it difficult to extract them using aqueous solutions. This might be the reason for the fact that a high amount of loaded protein was required to detect bands for SV2b and synaptophysin in the hippocampal extract. A high amount of loaded protein can result in non-specific binding of the antibodies and consequently to a high background, and the intensity of the bands will vary from experiment to experiment. Therefore, in the present thesis data for SV2b and synaptophysin in the hippocampal formation were excluded. Furthermore, some glycoproteins such as SV2b require careful denaturing procedures before being loaded onto the SDS gel. Upon denaturation, SV2b protein forms aggregates and displays a smear like pattern on the gel/blot which is common for this protein (Bajjalieh et al., 1994). In the present thesis, conditions of protein denaturation were optimized to reduce the smear-like pattern, however, the slight tailing of the band representing SV2b could not be completely avoided.

4.1.4 The chronic stress paradigm as model of depression

The resident intruder paradigm is a model of social defeat and subordination (Koolhaas et al., 1990) that is regarded as preclinical model of depression (Fuchs and Flügge, 2002). Several models of stress-induced depressive-like symptoms have been used to investigate neurobiological mechanisms underlying depression, particularly those models that mimic every day life (Willner et al., 1995; Willner, 1995; 1997; Willner and Mitchell, 2002). In the present thesis, the resident intruder paradigm was used to induce depressive-like symptoms in rats in order to study modulatory effects of stress and CIT on gene expression. In a preceding study, chronically stressed rats showed behavioural changes, including

decreased motility and reduced exploratory activity, increased immobility in the forced-swim test, and reduced preference for sucrose solution (Rygula et al., 2005). Reduced locomotor and exploratory activity represents a loss of interest in a new environment, implying a deficit in motivation which is a key feature of depression in humans. Increased immobility in the forced-swim test indicates behavioural despair which is also characteristic for depressive disorders. Decreased sucrose preference may indicate desensitisation of the brain reward mechanism and is indicative of anhedonia, another core symptom of depression in humans (DSM-IV, 1994). This chronic social stress model in rats has already been validated as model to study effects of antidepressants in that the chronic oral administration of citalopram abolished the stress-induced behavioural changes and normalized behaviours related to motivation and reward sensitivity (Rygula et al., 2006). The present data further substantiate the validity of the chronic social stress paradigm as a model to study central nervous molecular mechanisms underlying a depressive-like state. Data reveal that effects of CIT in stressed animals differ from those in unstressed rats and it is known that in humans, antidepressants are only effective in depressed but not in healthy subjects. Therefore, to elucidate molecular mechanisms of antidepressant action, it appears important to induce a depressive-like state in the animals prior to the treatment with an antidepressant drug.

4.1.5 The antidepressant citalopram

Citalopram is a highly selective and potent 5-HT reuptake inhibitor with minimal effects on the neuronal reuptake of norepinephrine and dopamine (Hyttel, 1977). Therefore, CIT can be regarded as the drug of choice to investigate effects of a SSRI on gene expression in the DRN, the brain area that contains the serotonergic neurons which project to the forebrain. The ability of CIT to potentiate serotonergic activity in the central nervous system via inhibition of the neuronal reuptake of serotonin is thought to be responsible for its antidepressant action. CIT is water soluble and can, therefore, be easily administered via the drinking water. The elimination half life of CIT from the blood ($t_{1/2}$) is approximately 37 hours (range 30-42 hours, Hyttel et al., 1984). CIT is metabolized in the liver to demethylcitalopram (DCIT), di-

demethylcitalopram (DDCIT) and citalopram N-oxide (Hyttel, 1977; Hyttel et al., 1984). It has been shown that DCIT, DDCIT and citalopram-N-oxide also inhibit the neuronal reuptake of serotonin but are less selective and less potent than the parent compound and are of minor clinical importance (Hyttel, 1977; Hyttel et al., 1984). Unchanged citalopram is the predominant compound in plasma of humans treated with the drug.

The therapeutic level of CIT in human blood is 30-130 ng/ml (Baumann et al., 2004). To attain this level in the present study in rats (the 'therapeutic level' of CIT in rats not unknown), CIT was administered at a dose of 30 mg/kg based on results obtained in a pilot study as described before (Rygula et al., 2006). This dose appears quite high, since patients are normally receiving a daily dose between 20 to 60 mg per individual. However, it is well known that the metabolic rate of drugs in rats is much faster than in humans, thus, it is not surprising that the dose measured in mg/kg for rat is often identical to a single dose per human individual.

The pharmacotherapy of depression typically requires chronic drug treatment. To obtain a realistic antidepressive intervention in the present experiments, the CIT treatment was begun when the stress-induced behavioural changes were already obvious (Rygula et al., 2005; 2006). The drug was then given daily for the clinically relevant period of four weeks while maintaining chronic social stress.

4.2 Potential evidence of stress-induced facilitation of neurotransmission and normalization by citalopram in the DRN

SV2b is involved in neurotransmitter release in that it regulates the readily releasable pool of synaptic vesicles and their exocytosis (Janz et al., 1999; Xu and Bajjalieh, 2001). It forms a complex with synaptotagmin 1 and has been implicated in modulation of intracellular calcium upon depolarization (Lazzell et al., 2004; Janz et al., 1999). In the present study, SV2b was found to be upregulated by chronic stress and normalized by CIT at the mRNA and protein level. These alterations in SV2b levels

presumably lead to modulation of synaptic vesicles exocytosis. Enhanced SV2b expression as it occurs after chronic stress could reflect facilitation of neurotransmitter release, which is normalized by the antidepressant. In line with this hypothesis, it was found that stress also increased SNAP-25 expression both on the mRNA and the protein level as shown by the *t*-test comparing just the data from the stress and the control group. In the course of the fusion process with the terminal plasma membrane, vesicle proteins interact with distinct terminal plasma membrane proteins, which leads to formation of an intermediate complex that is stabilized by SNAP-25 (Bock and Scheller, 1999; Chen et al., 1999; Fiebig et al., 1999; Sollner et al., 1993). SNAP-25 was shown to play a role in axonal growth *in vitro* and there are indications that high levels of this protein in specific regions of the adult brain contribute to nerve terminal plasticity (Osen-Sand et al., 1993). CIT did not normalize SNAP-25 mRNA expression although SNAP-25 protein expression in stressed animals treated with CIT showed a trend towards normal, however, with a high standard deviation. This may be related to the fact that SNAP-25 protein is not only found at the terminal membrane but is also associated with other membranes within neurons (Verderio et al., 2004). In unstressed animals, CIT even evoked a non-significant increase in SNAP-25 mRNA which coincides with findings showing an upregulation of SNAP-25 in the hypothalamus of unstressed rats after chronic imipramine and St. John's wort administration (Wong et al., 2004).

In the present thesis, to test whether stress and CIT may have a global effect on proteins related to synaptic functions, expression of the synaptic terminal protein syntaxin 1A and of the synaptic-vesicle protein synaptophysin was also investigated. Data show that expression of both proteins is not changed by chronic stress and CIT in the DRN. Therefore, one may speculate that only SNAP-25 and SV2b might play distinct roles in stress-induced facilitation of neurotransmission. Their stress-induced upregulation supports the hypothesis that chronic stress leads to presynaptic changes in DRN neurons, possibly reflecting increased 5-HT neurotransmission. It has been reported that stress increases the 5-HT level in the DRN (Maswood et al., 1998; Amat et al., 2005) and that different stressors, including psychological stress, raise 5-HT levels in target areas of this nucleus (Kirby et al., 1997; Kawahara et al., 1993). However, changes in expression of SV2b/SNAP-25 might have occurred in serotonergic as

well as non-serotonergic neurons because these proteins are expressed in different types of neurons (Oyler et al., 1992; Bajjalieh et al., 1994). However, since approximately 30% of the cells in the DRN are serotonergic (Descarries et al., 1982) one can assume that the increase in the expression of SNAP-25/SV2b mRNA occurs at least in part in serotonergic neurons. Extracellular 5-HT in the DRN is derived from local neurons that display vesicle-containing dendrites (Descarries et al., 1982), dendro-dendritic synapses (Chazal and Ralston, 1987) and collaterals of serotonergic axons (Beaudet and Descarries, 1981), which suggests the presence of SNAP-25 and SV2b proteins in the serotonergic neural dendrites and terminals of the DRN. Moreover, there is evidence that SNAP-25 (Verderio et al., 2004) and SV2b (Bajjalieh et al., 1994) may not be expressed in GABA-ergic neurons, which are abundant in the DRN (Fonnum and Walberg, 1973; Ribak et al., 1976). However, it remains to be elucidated whether the changes in the analyzed transcripts occurred in the serotonergic or in non-serotonergic neurons of the DRN and whether the presumptive changes in neurotransmission have consequences for 5-HT release in target regions where these neurons project to.

4.3 Chronic stress and citalopram effects on genes related to serotonin neurotransmission in the DRN

The concept that stress/antidepressants modulate the serotonergic system would imply alterations in expression of genes directly related to regulation of 5-HT neurotransmission in the DRN. Stress is known to activate the 5-HT system meaning that biosynthesis, release and feed-back action of this monoamine are affected. Effects of the SSRI could be mediated directly via binding to SERT, or indirectly via the feed back inhibition evoked by 5-HT_{1A} autoreceptor stimulation. However, sequences of genes directly related to serotonergic mechanisms could not be found in the cDNA library after subtractive hybridization suggesting that expression of those genes is not changed after five weeks of social stress, or that the alterations in their expression cannot be detected by SH. To verify whether

expression of genes related to 5-HT neurotransmission was really unaffected by the stress and the CIT treatment quantitative real-time PCR was performed. No significant alteration in SERT, TPH2 and 5-HT_{1A} autoreceptor transcripts expression was detected, but stress induced an approximately 3.5-fold increase in TPH1 mRNA. Regarding the lack of response in the mRNA of SERT, TPH2 and 5-HT_{1A}, there might be two explanations: First, expression of these molecules is really not regulated by the social stress, or second, expression was regulated by stress but the effect disappeared in the course of the chronic stress period. However, in another chronic social stress paradigm using a non-rodent species, the male tree shrew (Fuchs and Flügge, 2002), a receptor binding study with the radiolabeled agonist 8-OH-DPAT showed no change in 5-HT_{1A} autoreceptor number in the DRN at different time points in the course of a 4-weeks social stress period, but only a decrease in affinity of the autoreceptors (Flügge, 1995). This may support the first hypothesis that there is no transient change in 5-HT_{1A} receptor expression during chronic social stress. In coincidence with the observations in tree shrews, desensitization of 5-HT_{1A} autoreceptors (reduced responsiveness to agonists) was also observed after 8 weeks of chronic mild stress in mice (Lanfumey et al., 1999), and after uncontrolled environmental stress in rats (Laaris et al., 1997). Altogether, these findings suggest that in the serotonergic neurons of the DRN, the response of 5-HT_{1A} autoreceptors to stressful events is restricted to changes in receptor affinity resulting in altered agonist sensitivity, but does not include alterations in expression of 5-HT_{1A} autoreceptor transcripts. CIT application decreased 5-HT_{1A} autoreceptor mRNA only in the DRN of the *Stress CIT* animals, again indicating that the antidepressant acts in a distinct manner in subjects exposed to aversive life events compared to unaffected controls. The lack of CIT effect on mRNA for the 5-HT_{1A} receptor in the *Control CIT* group coincides with a previous study performed by Spurlock and colleagues (1994). One may speculate that antidepressant induced changes in 5-HT_{1A} autoreceptor sensitivity in otherwise untreated rats did not occur on the mRNA level (Kreiss and Lucki, 1995; Le Poul et al., 1995; Hanoun et al., 2004; El Mansari et al., 2005). However, SSRIs may have transient effects on 5-HT_{1A} receptor gene expression as mRNA was found to be significantly decreased after 2-3 weeks of fluoxetine treatment in the anterior raphe of rats (Le Poul et al., 2000).

The 5-HT_{1A} autoreceptors are G-protein coupled receptors which are negatively coupled to adenylyl cyclase and thus inhibit cAMP formation (Yocca et al., 1986). Upon stimulation, these autoreceptors decrease the firing rate of 5-HT neurons (Aghajanian et al., 1972). This inhibition of neuronal activity is mediated by the interaction between the G-protein and ion channels, which increases potassium conductance (Aghajanian and Lakoski, 1984) and reduces the high threshold calcium current (Penington and Kelly, 1990). The later effect leads to reduction in the amount of 5-HT which is released upon depolarization (see Pineyro and Blier, 1999). Continuous stimulation of the autoreceptors may affect 5-HT release also by inhibiting its biosynthesis (see below). The observed reduction in 5-HT_{1A} mRNA in the *Stress CIT* group was possibly due to the combination of increased 5-HT release due to the chronic stress and due to 5-HT reuptake inhibition. It has been previously demonstrated that various stressors increase 5-HT levels in the DRN and different brain regions (Kawahara et al., 1993; Kirby et al., 1997; Maswood et al., 1998; Amat et al., 2005). These data may reflect that mRNA for the extrasynaptically located 5-HT_{1A} autoreceptor in the DRN is downregulated upon convergence of intracellular pathways stimulated by high extracellular 5-HT levels induced by stress and CIT treatments.

Expression of SERT was also found to be unchanged in the DRN after 5 weeks of social stress in the rat DRN. However, in mice, 10 days of social defeat upregulated SERT mRNA in a larger brain stem region defined as 'area of raphe nuclei' (Filipenko et al., 2002). It is possible that the difference between these and the present data is due to the fact that in the present study, the DRN was selectively punched out for isolation of transcripts. In this context it is interesting to note that after acute immobilization stress in rats, SERT mRNA expression was found to be unchanged in the DRN but was downregulated in the raphe pontis (Vollmayr et al., 2000). On the other hand, no significant effects of CIT on SERT mRNA expression were detected in any of the animal groups in the present study. This finding is in line with data by Spurlock et al. (1994) who found no effect of different antidepressants on SERT gene transcripts in brains of rats treated chronically for up to 32 days indicating that expression of this gene is relatively stable. However, SERT mRNA was transiently reduced in the DRN of rats treated with

fluoxetine after 7 days, an effect that disappeared after 21 days (Neumaier et al., 1996). Therefore, one may speculate that antidepressants change the expression of SERT transcripts in a transient manner. It should also be considered that a common polymorphism within the transcriptional control region of the SERT gene has been detected in patients with depressive disorders that may have an impact on efficacy of antidepressant drugs (Lesch and Gutknecht, 2005).

Stress elevates 5-HT levels in the DRN (Maswood et al., 1998; Amat et al., 2005) and in its target areas (Kawahara et al., 1993; Kirby et al., 1997) which might be related to increased expression of tryptophan hydroxylase (TPH), the rate limiting enzyme of the 5-HT biosynthesis pathway. Moreover, it is long known that stress increases TPH enzyme activity (Azmitia and McEwen, 1974). Several studies reported an increase in serotonin production and turnover after chronic stress (see Flügge, 2000). In the initial experiments of the present thesis, expression of mRNA for the first described TPH gene, now known as TPH1 (Darmon et al., 1988) was quantified. Real-time PCR data revealed that TPH1 mRNA was upregulated by stress and normalized by CIT in *Stress CIT* animals, while the drug increased TPH1 mRNA in *Control CIT* animals. These findings coincide with data on TPH1 mRNA and TPH protein expression detected after chronic immobilization stress in the rat DRN (Chamas et al., 1999; 2004). The gene for TPH2 has recently been described for vertebrates being the predominantly expressed gene in the brain stem responsible for 5-HT biosynthesis (Walther et al., 2003; Zhang et al., 2004). In light of those data on TPH2 it has been proposed that studies attempting to link TPH1 with the actions of antidepressants and mood disorders have to be reconsidered (see Serretti and Artoli, 2004). As shown here, TPH2 mRNA expression was unchanged after four weeks of social stress, but CIT reduced TPH2 mRNA in both stressed and unstressed rats. One may speculate that this low expression level might be caused by an inhibitory feed back mechanism induced by high 5-HT as a result of its extracellular accumulation due to reuptake blockade. However, changes in expression of TPH protein induced by stress and CIT did not correlate directly with alterations in mRNA for one of the TPH genes. Instead, it appears that alterations in transcripts from both genes contribute to the effects of stress and CIT on TPH protein, in that TPH1 mRNA is upregulated by stress whereas TPH2 mRNA is downregulated by

CIT in all animals irrespective of a concomitant stress exposure. These data indicate that both genes, TPH1 and TPH2, should be considered when studying molecular mechanisms of stress and antidepressant action on 5-HT biosynthesis in the DRN. It has been reported that TPH expression and activity is reduced concomitantly with a reduced neuronal firing rate upon stimulation of 5-HT_{1A} receptors (Fernstrom et al., 1990; Invernizzi et al., 1991; Florez and Takahashi, 1996). Therefore, there might be an interrelation between 5-HT_{1A} autoreceptor stimulation and 5-HT biosynthesis, possibly via the intracellular cAMP signalling pathway.

Elevated TPH protein expression may correlate with increased 5-HT synthesis as it is known that stress raises the concentration of this neurotransmitter and its metabolites in target brain regions (Azmitia and McEwen, 1974; Thierry et al., 1968; Raab, 1971; Palkovits et al., 1976; Kirby et al., 1997; Chaouloff, 1993; 2000). However, there are also reports saying that chronic stress reduces 5-HT in target regions of the DRN (Mangiavacchi et al., 2001) indicating that chronic exposure to challenging events might finally lead to a deficit with respect to the capability of 5-HT neurons to synthesize sufficient amounts of the neurotransmitter when 5-HT release is persistently accelerated. The findings of a stress-induced upregulation of proteins involved in neurotransmitter release, SV2b and SNAP-25, indicate that DRN neurons are indeed more active.

4.4 Chronic stress and citalopram effects on genes involved in neuronal survival and neuroplasticity in the DRN

CREB binding protein (CBP) is a conserved transcriptional adaptor protein which contains histone acetyltransferase (HAT) activity that links transcription with chromatin remodeling (Goodman and Smolik, 2000). HATs catalyze the acetylation of selected positively charged amino acids in the histone tails (lysine and arginine) which neutralizes these charges and causes chromatin unfolding. This process facilitates binding of transcription factors to DNA and thus modulates gene transcription

(Meaney and Szyf, 2005; Goodman and Smolik, 2000). One can assume that alterations in HAT expression have an impact on gene expression. A presumptive involvement of serotonin and the HAT CBP in regulation of transcription induced by external signals triggering the intracellular cAMP signaling pathway has been proposed (see Meaney and Szyf, 2005). CBP is of particular interest because it is important for recruitment of the transcription factor CREB that displays neuroprotective functions (Mayr and Montminy, 2001; Lonze and Ginty, 2002; Lonze et al., 2002). CREB-directed gene transcription has been shown to be regulated by antidepressants (Schwaninger et al., 1995). CREB itself has been shown to be regulated by stress and antidepressants and its role in the pathophysiology of mood disorders has been demonstrated by *post mortem* studies (Alfonso et al., 2006; Nibuya et al., 1996; Dowlatshahi et al., 1998, respectively). To my knowledge this study is the first showing that also CBP expression is regulated by stress/antidepressants. One may speculate that CIT-mediated normalization of CBP mRNA expression may represent an initial step in mechanisms that restore normal gene transcription. Future studies are required to investigate whether the stress-induced increase in CBP expression and CIT modulation as detected in the present study might be related to CREB activity in the DRN.

Expression of neuron-specific enolase (NSE) was found to be regulated by stress and CIT. NSE is the $\gamma\gamma$ -isozyme of enolases that are glycolytic enzymes composed of two subunits. NSE is found in mature neurons and neuroendocrine cells (Marangos et al., 1980; Marangos and Schmechel, 1987). It has been reported that this isozyme has neurotrophic and neuroprotective effects on different types of cultured neurons (Takei et al., 1991; Hattori et al., 1995). The present study shows that in comparison to controls, expression of both NSE mRNA as well as NSE protein was elevated in stressed animals possibly reflecting accelerated metabolism in DRN neurons in response to the chronic social stress. One could have expected that CIT reversed this stress-induced increase, but in contrast, NSE mRNA was further upregulated in both *Stress CIT* as well as *Control CIT*. NSE protein showed the same elevated level of expression in all CIT treated animals. Based on the presumptive neurotrophic functions of NSE, one may assume that enhanced expression of this enolase represents a supportive mechanism

exerted by neurons in response to stress and CIT. This interpretation is in line with the view that antidepressants stimulate supportive neuronal plasticity within the neural system (Duman et al., 1997; 1999). However, the molecular mechanisms underlying the neurotrophic and neuroprotective activities of NSE are still unknown and require further investigations.

The present data are in line with the view that chronic stress induces neuronal remodeling as shown for the hippocampus of tree shrews and rats, where chronic stress induced a retraction of apical dendrites of pyramidal neurons (Magarinos and McEwen, 1995; Magarinos et al., 1996), and reorganization of synaptic structures at mossy fiber terminals in rats (Magarinos et al., 1997). Examples of molecular mechanisms underlying these structural changes in hippocampal pyramidal neurons are the stress-induced downregulation of membrane glycoprotein M6a and changes in expression of other genes known to be involved in neuronal plasticity (Alfonso et al., 2004a; 2005). However, presumptive structural changes in DRN neurons related to the present changes in gene expression require further investigations.

4.5 Regulation of NDRG2 expression: Indication for glia-mediated plasticity in the DRN

NDRG2 is a member of the N-myc downregulated gene (NDRG 1-4) family with putative roles in neural differentiation, synapse formation and axonal survival in the developing and adult nervous system (Nichols, 2003; Nichols et al., 2005). It is expressed in astrocytes and was found to be upregulated in the hippocampus of adrenalectomized rats in response to corticosterone treatment (Nichols and Finch, 1994; Nichols, 2003; Nichols et al., 2005). In the frontal cortex of unstressed rats, two splice variants of NDRG2 mRNA and protein were downregulated by sertraline, imipramine and electroconvulsive treatment, respectively (Takahashi et al., 2005). In the present thesis, NDRG2 mRNA was upregulated after chronic stress in the DRN, and CIT normalized NDRG2 mRNA expression in *Stress CIT* animals

but had no effect in *Control CIT* subjects. The discrepancy between those data and the data from Takahashi et al. (2005) indicates that effects of antidepressants on gene expression differ between brain regions. The impact of stress and antidepressants on NDRG2 regulation supports the recently emerging hypothesis that astrocytes are involved in stress-related pathologies and mood disorders. For example in tree shrews, five weeks of social stress decreased the number and the volume of astroglial cells in the hippocampus (Czeh et al., 2005), whereas in rats an increased density of GFAP-immunoreactive astrocytes was detected in the hippocampus after one week of stress (Lambert et al., 2000). GFAP is expressed in a subpopulation of astrocytes that are known to be essential for proper neuronal functioning and synaptic transmission (Newman, 2003). Interestingly, *post mortem* studies in humans indicated a central role of glial cells in psychiatric disorders (Ongur et al., 1998; Cotter et al., 2002; Stark et al., 2004). The present findings showing CIT induced normalization of NDRG2 mRNA expression support the view that the therapeutic actions of antidepressants are not restricted to neurons but include also astrocytes.

4.6 Other stress-regulated genes in the DRN

After subtractive hybridization, the cDNA libraries of potentially stress-regulated genes contained gene sequences for extracellular matrix proteins, namely procollagen, chondroitin sulphate proteoglycan and osteonectin. Alterations in extracellular matrix components occur in the context of synaptic plasticity, in inflammatory processes and may be associated with brain pathologies including dementia-associated diseases (Rauch, 2004). Some of the ECM components may affect neural plasticity and cellular functions by binding to other ECM elements such as osteonectin (Sweetwyne et al., 2004). Chondroitin sulfate proteoglycans may influence neural plasticity through binding to cell surface molecules (Maurel et al., 1994). However, within the time period of this thesis, it was not possible to confirm regulatory effects on the above genes by real-time PCR and Western blot analysis.

The cDNA libraries of stress regulated genes also contained sequences for mitochondrial genes. A presumptive increase in expression of mitochondrial genes could represent auxiliary mechanisms in order to increase energy availability for the neurons in response to the continuous energy demand, e.g. in the context of enhanced neurotransmitter release. It has been shown that in the hippocampus, mossy fiber terminals that are occupied by a high number of mitochondria are enlarged after chronic stress (Magarinos et al., 1997). Also the 'stress-hormones' cortisol and corticosterone regulate expression of mitochondrial genes in the hippocampus when administered chronically (Alfonso et al., 2004b; Datson et al., 2001). It is interesting to note that in addition, chronically administered antidepressants increased mRNA levels of the mitochondrial gene for cytochrome b in mouse cerebral cortex (Huang et al., 1997) and cytochrome c oxidase mRNA in the rat hippocampus (Drigues et al., 2003). The observation that effects of chronic stress and antidepressants on expression of certain mitochondrial genes go in the same direction may support the hypothesis that both conditions are accompanied by increased energy demands in neural tissue (Wong et al., 2004).

Furthermore, genes related to processes of protein synthesis and degradation that are important for vital cellular functions were contained in the cDNA libraries that were generated in this thesis. The ribosomal proteins S29, L37, 40S and S8 were contained in the library of potentially upregulated genes, while ribosomal protein S9 was found in the library of potentially downregulated genes. Stress could affect the recruitment of these proteins in order to stabilize the configuration of the ribosomal RNAs to improve protein synthesis, which is crucial for several cellular functions, for cell proliferation and neurogenesis (Wool, 1996). Ribosomal proteins were shown to be regulated by chronic cortisol administration in the hippocampus of tree shrews (Alfonso et al., 2004a; b) and in the hippocampus of adrenalectomized rats under different conditions of corticosteroid exposure (Datson et al., 2001).

4.7 Effects of stress and citalopram on expression of NSE protein and synaptic components in the hippocampus

NSE is regarded as neurotrophic and neuroprotective factor and has been reported to be involved in cell growth, proliferation and survival (Hattori et al., 1995; Brady and Lasek, 1981). In the hypothalamus of rats treated chronically with antidepressants, NSE was found to be upregulated (Wong et al., 2004). The present study shows that like in the DRN, NSE protein is also upregulated by chronic stress in the hippocampus. According to its presumptive functions changes in NSE expression might reflect a supportive or adaptive response exerted by neurons under stressful events. In contrast to the findings in the DRN, CIT had no effect on NSE protein expression in the hippocampus. This means that the antidepressant CIT modulates expression of the NSE protein in a region specific manner.

Effects of stress and CIT on proteins related to synaptic function, whose expression was regulated in the DRN were also investigated in the hippocampal formation. Expression of the terminal membrane protein SNAP-25 protein was only upregulated by chronic CIT in the *Control CIT* group. It is interesting to note that in the hippocampus, expression of the other terminal membrane protein, syntaxin 1A, was upregulated by stress and normalized by CIT thus revealing a regulatory pattern resembling that of SNAP-25 in the DRN. CIT also reduced syntaxin 1A protein in the *Control CIT* group. Although the underlying molecular processes are still unknown one may speculate that depending on the brain region, different proteins involved in similar cellular functions might be affected by stress and/or the SSRI. The present data support and extend previous findings showing that antidepressants do regulate synaptic proteins, but the effects are region and/or drug specific (Rapp et al., 2004; Yamada et al., 2002; see Alfonso et al., 2005).

SV2b and synaptophysin expression in the hippocampus was also investigated, but unfortunately the intensity of the respective bands in the blots was too low to be quantified although up to 80 µg total proteins per lane was loaded on the gels. The reason for this can be attributed to inherent problems associated with Western blot and/or the protein itself (see Methodological aspects).

4.8 Summary and conclusions

4.8.1 Chronic stress and citalopram regulate expression of genes involved in synaptic functions and neuroplasticity

In agreement with previous studies about effects of chronic stress and antidepressants treatments in the hippocampal formation (for review see Alfonso et al., 2005), the present data indicate that stress and the SSRI citalopram modulate expression of genes involved in synaptic functions, neuronal survival and protection, neuronal plasticity as well as glial functions. Stress-induced upregulation of the terminal membrane protein SNAP-25 and of the synaptic vesicle protein 2b may reflect facilitation of neurotransmitter release mechanisms coinciding with the view that stress activates DRN neurons. One may speculate that the CIT induced normalization of SV2b expression reflects an at least partial restoration of normal release processes. It remains to be determined whether these regulatory processes affect all DRN neurons or only a subpopulation, e.g. the serotonergic neurons. However, these are regional effects of stress and CIT as data on gene expression in the hippocampus differed from those in the DRN.

Stress-induced upregulation of the glial gene NDRG2 reveals that in the DRN, glial cells are also affected by stress and that CIT may normalize the effect. This finding coincides with previous data by Czeh et al. (2005) showing that in the hippocampal formation, stress and antidepressants affect the number of glial cells.

The observed changes in the expression of CBP, the protein that enables the transcription factor CREB in its phosphorylated form to bind to DNA by unfolding chromatin, indicate that stress also affects chromatin structure and that CIT counteracts this effect. Furthermore, upregulation of neuron specific enolase, the glycolytic enzyme that has been previously supposed to be involved in neuroplasticity, may reflect that DRN neurons display neuroplastic changes in chronic stress. It is interesting to note that expression of NSE was upregulated by both stress and CIT, which possibly reflects increased energy demands under both conditions, stress and antidepressant treatment.

4.8.2 Chronic stress and citalopram modulate 5-HT biosynthesis

An important finding of the present thesis is that CIT counteracts the stress-induced upregulation of TPH protein which strongly indicates that besides blocking 5-HT reuptake, the SSRI also normalizes 5-HT biosynthesis. Furthermore, it is a new finding that the two TPH genes that are known today, TPH 1 and 2, are differentially regulated by stress and CIT on the mRNA level whereas the concentration of total TPH protein is finally upregulated by stress and entirely normalized by CIT. The downregulation of 5-HT_{1A} autoreceptor expression that is induced by CIT may lead to reduced negative feed-back inhibition and thus to facilitation of 5-HT release. This mechanism may further increase extracellular 5-HT, in addition to the 5-HT elevation mediated by the serotonin transporter blockade. Altogether, these effects may represent a new homeostatic state induced by CIT in the DRN of chronically stressed subjects. One may speculate that similar mechanisms may underlie the therapeutic actions of CIT in patients.

4.8.3 Stress models to study molecular mechanisms of antidepressants

It is known that stressful life events may trigger the onset of major depression (Kendler et al., 1999; McEwen, 2002). In animal models, stress-induced neurochemical/neuromolecular alterations (Czeh et al., 2001; McEwen et al., 2002; Alfonso et al., 2004a) and behavioural changes such as anhedonia (Rygula et al., 2006) are reversed by chronic antidepressant treatments. The present study supports the view that these models are valid tools to investigate molecular actions of antidepressants. It was found that a chronic treatment with the SSRI CIT normalizes expression of target genes in the DRN, the primary central nervous site of action of SSRIs. Data from the present study thus contribute to a better understanding of the molecular mechanisms underlying the antidepressive effects of SSRIs.

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Publications

Articles in peer-reviewed journals

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Courses taken during the Ph.D. program

"Scientific communication". Instructor: Dr. H. Silyn Roberts, Gttingen, Germany. January, 2001.

"Real-time PCR and gene expression analysis". IBA academy in Gttingen, Germany. June, 2003.

"Codelink 3D-microarray and gene expression seminar". Max Planck Institute for experimental Medicine in Gttingen, Germany. November, 2003.

"HNB-bioinformatics course". Seminar and training course in DKFZ, Heidelberg, Germany. November, 2003.

"Introduction to statistical analysis of Microarray data". Course Lecturers: Prof. Dr. E. Brunner and Dr. J. Landgrebe, Department of Biostatistics, University of Gttingen, Germany. March, 2004.

"Best practice in Microarray studies". Universittsklinikum Heidelberg, PD Dr. U. Mansmann, Heidelberg, Germany. December, 2004.

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